PATENT
2798-1-001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Alfonso Fernández-Mayoralas Alvarez Examiner : Underdahl, Thane E.

Serial No.: 10/738,378 Group Unit : 1651

Filed: December 17, 2003

For: ENZYMATIC METHOD OF PRODUCING 4-O-β-D-GALACTOPYRANOSYL-D-XYLOSE, 4-O-β-D-GALACTOPYRANOSYL-D-XYLOSE OBTAINED USING SAID METHOD, COMPOSITIONS CONTAINING SAME AND THE USE THEREOF IN EVALUATING INTESTINAL LACTASE

DECLARATION UNDER 37 C.F.R. 1.132

COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Alfonso Fernández-Mayoralas Alvarez, as evidenced by my signature below, declare the following:

1. I received a Ph.D. in Chemistry in 1987 from Autónoma University of Madrid (Spain). I received a Master's Degree in Chemistry in 1983 from Autónoma University of Madrid. I received a Bachelor's Degree in Chemistry in 1982 from Autónoma University of Madrid.

2. In addition to the academic appointments set forth in paragraph 1, *supra*, I was a Research Scientist at various public institutions (see Exhibit A) from 1985 - 2007.

3. My research interests include developing synthesis processes for 4-galactosyl-xylose. A detailed chronology of my academic and professional accomplishments is submitted herewith in my *Curriculum Vitae*, Exhibit A.

4. I have reviewed the above-referenced United States patent application, Serial Number 10/738,378, the subject matter described therein as well as the currently pending claims in the application and Reyes *et al.,* U.S. Patent 5,994,092, Ponpipom *et al.,* U.S. Patent 4,228,274, Crumpton *et al., Biochem. J.* 70(4):729 (1958), Wong-Madden *et al.,* U.S. Patent 5,770,405, Dahmen *et al.,* U.S. Patent 4,675,392, Rao *et al., Qual. Plant.-Pl. Fds. Hum. Nutr. XXVIII* 4:293-303 (1979), Gabelsberger *et al., FEMS Letters* 109(2-3): 131 (1993), Fujimoto *et al., Glycoconjugate Journal* 15:155 (1998) and Yoshitake *et al., Eur. J. Biochem.* 101:395 (1979).

5. The claims pending in the above-referenced patent application are directed to obtaining a product useful for evaluating intestinal lactase. In the process described two parts can be distinguished, namely an enzyme reaction and a subsequent purification of the reaction mixture. Developing a method for purifying a carbohydrate mixture is neither easy nor routine for one skilled in the art, due at least in part to the characteristics of carbohydrate molecules.

6. The chemistry of carbohydrates is very complex, since different carbohydrate molecules have very similar structures. Many carbohydrates even have the same molecular formula. For example, lactose and sucrose both have 12 carbons, 22 hydrogens and 11 oxygens and the same type of functional group (the hydroxyl group).

7. Small changes in the structure of carbohydrates (for example, two carbohydrates that differ in the stereochemistry of one of their chiral centres, such as celobiose and lactose, can give rise to significant differences both in chemical reactivity and in behavior in the purification processes. Raymond Lemieux opined the following: *"The only generalization that*

exists in the chemistry of carbohydrates is that there is no generalization. "

8. Applicants submit herewith two additional journal articles that each further describe some of the complexities and problems associated with carbohydrate chemistry, Marcaurelle *et al., Current Opinion in Chemical Biology*, 2002, 6:289-296 (Exhibit B) and Holemann *et al., Current Opinion in Biotechnology*, 2004, 15:615-622 (Exhibit C).

9. Although crystalization is in fact a common process for purifying sugars, finding the appropriate solvent is not easy. The appropriate solvent depends on the type of molecules and the range of solvents that must be tested can be very broad. The more customary solvents in sugars tend to be low molecular weight alcohols, water, ethyl acetate, hexane, and their mixtures. In a crystallization process, a large number of solvents and mixtures thereof must be tested or screened before arriving at the appropriate solvent to use. In the case of the above-referenced patent application, acetone allows obtaining the product desired with a >99% degree of purity, which was not possible with more usual solvents. In Exhibit D a gas chromatogram of the 4-galactosyl-xylose obtained by the process described in the above-referenced patent application, can be shown. Peaks at retention times of 18.70 and 18.92 min correspond to alpha and beta anomers of 4-galactosyl-xylose, respectively. By simply summing the % areas of each peak (92,566 + 6,540 = 99,106%), a purity of over 99% is achieved.

10. Wong-Madden *et al.,* U.S. Patent 5,770,405 do not use the solvent mixture in a chromatography on active carbon. Wong-Madden *et al.* teach using isopropanol/ethanol/water, but it is to develop a chromatography on silica gel. (See, Column 33, line 25).

11. Active carbon is customarily used to eliminate hydrophobic impurities, but it is not normally used in organic synthesis, for separating monosaccharide and disaccharide mixtures, such as is the case in the above-referenced patent application. The normal course to separate these mixtures is to employ chromatography on a silica gel, on sepharose or others (*See,* Wong-Madden et al., Column 11, line 19). The above-referenced patent application

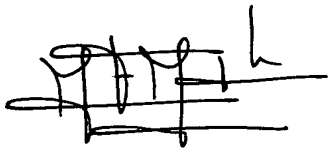
describes purifying a mono- and disaccharide mixture using active carbon, which offers the advantage, compared with usual adsorbents (e.g., silica gel or sepharose) of being cheaper. In H. Rotzche, *Journal of Chromatography Library* 1991, 48:104-107 (Exhibit E), either structural and geometrical differences between each kind of adsorption matrixes, active carbon in comparison with other column fillings as polymers, silica gel, etc. are discussed in detail.

12. The above-referenced patent application describes an isopropanol/water mixture as eluent, as opposed to the more common alcohol/water mixtures such as methanol/water or ethanol/water. The methods described in the above-referenced patent application thereby provide the advantage of allowing for less elution volume, a significant advantage for industrial production (Exhibit F).

13. Rao *et al.* teach extraction with Soxhlet to extract fats from a specimen of plant origin. Rao *et al.* do not describe using Soxhlet for selectively extracting monosaccharides from a mixture of sugars.

14. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, or any patent issuing thereon.

Submitted by



Alfonso Fernández-Mayoralas Alvarez

Date Signed: May 31, 2007

Exhibit A

CURRICULUM VITAE

Name: Alfonso Fernández-Mayoralas Alvarez

Date of Birth: August 1, 1960

Nationality: Spanish

Address: Institute of Organic Chemistry, C.S.I.C., Juan de la Cierva 3, 28006 Madrid.

1. ACADEMIC DEGREES

- Degree In Chemistry (BSC). Autónoma University of Madrid (Spain). 1982.
- Ph. D. In Chemistry. Autónoma University of Madrid. 1987. *Cum Laude*

2. PROFESSIONAL EMPLOYMENTS AND RESEARCH EXPERIENCE

- **1985-1987.** Graduent Student. Fellow of the MEC. Organic Chemistry Institute. CSIC. Supervisor: Dr. Manuel Martín-Lomas.
- **1988.** Postdoctoral Fellow, École Normale Supérieure, Paris. Supervisor: Dr. Pierre Sinaÿ
- **1989.** Posdoctoral Fellow. Massachusetts Institute of Technology. Supervisor: Dr. A.M. Klibanov
- **1990-2002.** Tenure Scientist at the Department of Biological Chemistry, Institute of Organic Chemistry, C.S.I.C. Madrid (Spain).
- **1991 (4 months).** Visiting Scientist at the Chemical Center, Lund (Sweden). Supervisor: Dr. K. G. I. Nilsson.
- **2002-.** Scientific Researcher at the Department of Biological Chemistry, Institute of Organic Chemistry, C.S.I.C. Madrid (Spain).

3. MAIN RESEARCH INTEREST

Carbohydrate chemistry. Synthesis of glycoconjugates with biological significance. Use of enzymes in organic synthesis.

4. PUBLICATIONS

A. Fernández-Mayoralas y M. Martín-Loma. Synthesis of 3-O-methyl and 4-O-methyltetra-O-acetyl-α-D-galactopyranose. *Anal. Quim.*, 80C (1984) 184-185.

A. Fernández-Mayoralas, M. Martín-Lomas y D. Villanueva. 4-O-β-D-Galactopyranosyl-3-O-methyl-D-glucose: a new synthesis and application to the evaluation of intestinal lactase. *Carbohydr. Res.*, 140 (1985) 81-91.

M. Alonso-López, M. Bernabé, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas y S. Penadés. Chiral macrocyclic compounds from lactose derivatives. *Carbohydr. Res.*, 150 (1986) 103-109.

A. Fernández-Mayoralas y M. Martín-Lomas. Synthesis of 3 and 2′-fucosyllactose and 3,2′-difucosyllactose from partially benzylated lactose derivatives. *Carbohydr. Res.*, 154 (1986) 93-101.

M. L. Jimeno, A. Fernández-Mayoralas, M. Martín-Lomas y A. Alemany. ^{13}C-NMR studies of peracetylated derivatives of O-α and O-β-D-galactopyranosyl-(1-->3) and (1-->4)-α-galactopyranose. *Carbohydr. Res.*, 161 (1987) 144-149.

M. Alonso-López, J. Barbat, E. Fanton, A. Fernández-Mayoralas, J. Gelas, D. Horton, M. Martín-Lomas, S. Penadés. The acetonation of lactose and benzyl β-lactoside with 2-methoxypropene. *Tetrahedron*, 43 (1987) 1169-1176.

S. David y A. Fernández-Mayoralas. Nouvelle voie d'acces a la configuration β-D-mannopyranoside protegee temporairement en position 3 et 6. *Carbohydr. Res.*, 165 (1987) C11-C13.

C. Jaramillo, A. Fernández-Mayoralas y M. Martín-Lomas. The acetonation of methyl β-maltoside with 2-methoxypropene. *Carbohydr. Res.*, 182 (1988) 153-158.

A. Fernández-Mayoralas, M. Bernabé, M. Martín-Lomas. The regioselectivity of dibutylstannylene mediated oxidation of methyl 3´,4´-O-isopropylidene-β and α-lactoside. A new synthesis of N-acetyllactosamine. *Tetrahedron*, 44 (1988) 4877-4882.

A. Fernández-Mayoralas, A. Marra, M. Trumtel, A. Veyrieres y P. Sinay. Convenient synthesis of substituted pyranoid glycals from thiophenyl glcosides and glycosyl phenylsulfones. *Tetrahedr. Lett.*, 30 (1989) 2537-2540.

A. Fernández-Mayoralas, A. Marra, M. Trumtel, A. Veyrieres y P. Sinay. Preparation of pyranoid glycal derivatives from phenyl thioglycosides and glycosyl phenylsulfones. *Carbohydr. Res.*, 188 (1989) 81-95.

M. Bernabé, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas y A. Rivera. The conformation of eight-membered 3,2´-O-isopropylidene acetals of some common disaccharides. *J. Chem. Soc., Perkin Trans. II*, (1989) 1867-1873.

T. Desai, A. Fernández-Mayoralas, J. Gigg, R. Gigg y S. Payne. The synthesis and resolution of 1,5,6-tri-O-benzyl-myo-inosito. *Carbohydr. Res.,* 205 (1990) 105-123.

T. Desai, A. Fernández-Mayoralas, J. Gigg, R. Gigg, C. Jaramillo, S. Payne, S. Penadés y N. Schnetz. Preparation of optically active myo-inosito derivatives as intermediates for the synthesis of inositol phosphates. *ACS Symposium Series*, Washington, DC (1991) 86-102.

E.Rubio, A. Fernández-Mayoralas y A. M. Klibanov. Effect of the solvent on enzyme regioselectivity. *J. Am. Chem. Soc.,* 113 (1991) 695-696.

E. Domínguez, J. C. Carretero, A. Fernández-Mayoralas y S. Conde. An efficient preparation of optically active (E)-γ-hydroxy-α,β-unsaturated phenylsulfones using lipase-mediated acylations. *Tetrahedr. Lett.*, 32 (1991) 5159-5162.

K. G. I. Nilsson y A. Fernández-Mayoralas. α-D-Galactosidase-catalysed synthesis of partially protected □-linked digalactopyranosides. *Biotechnol. Lett.*, 13 (1991) 715-720.

R. López, A. Fernández-Mayoralas, M. Martín-Lomas y J. M. Guisán. Enzymatic β-galactosidation of β-xylopyranosides. *Biotechnol. Lett.*, 13 (1991) 705-710.

A. Rivera-Sagredo, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas, D. Villanueva y J. J. Aragón. 4-O-β-D-Galactopyranosyl-D-xylose: A new synthesis and application to the evaluation of intestinal lactase. *Carbohydr. Res.*, 228 (1992) 129-135.

F. F. Santos-Benito, M. Nieto-Sampedro, A. Fernández-Mayoralas y M. Martín-Lomas. Synthesis of oligosaccharide inhibitors of neural cell division. *Carbohydr. Res.*, 230 (1992) 185-190.

R. López y A. Fernández-Mayoralas. Controlling yield and regioselectivity in the enzymatic synthesis of β-D-galactopyranosyl-β-D-xylopyranosides. *Tetrahedr. Lett.*, 33 (1992) 5449-5452.

F. F. Santos-Benito, A. Fernández-Mayoralas, M. Martín-Lomas y M. Nieto-Sampedro. Inhibition of proliferation of normal and transformed neural cells by blood group-related oligosaccharides. *J. Exp. Med.*, 176 (1992) 915-918.

T. Desai, A. Fernández-Mayoralas, J. Gigg, R. Gigg y S. Payne. The preparation of phosphorylated intermediates for the synthesis of racemic and chiral myo-inositol 1,4,5-trisphosphate and its phosphorothioimidate analogues. *Carbohydr. Res.,* 234 (1992) 157-175.

J. J. Aragón, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas, A. Rivera-Sagredo y D. Villanueva. Evaluation of rat intestinal lactase in vivo with 4-galactosylxylose. *Clin. Chim. Acta*, 210 (1992) 221-226.

R. López, C. Pérez, A. Fernández-Mayoralas y S. Conde.. Enzymatic transesterification of alkyl 2,3,4-tri-O-acyl-□-D-Xylopyranosides. *J. Carbohydr. Chem.*, 12 (1993) 165-171.

J. M. Guisán, V. Rodríguez, G. Soler, C. Santana, R. Fernández-Lafuente, A. Batisda, C. M. Rosell, R. López, A. Fernández-Mayoralas y M. Martín-Lomas. Synthesis of pharmaceutical oligosaccharides

catalyzed by immobilized-stabilized derivatives of different β-galactosidases. *J. Mol. Cat.*, 84 (1993) 373-379.

J. L. Asensio, R. López, A. Fernández-Mayoralas y J. Jiménez-Barbero. Conformational studies on β-galactopyranosyl-(1>3) and (1>4)-xylopyranosides by NMR, molecular mechanics, molecular dynamics, and semiempirical calculations. *Tetrahedron*, 50 (1994) 6417-6432.

K. Singh, A. Fernández-Mayoralas y M. Martín-Lomas. Synthesis of oligosaccharides structurally related to E-selectin ligands. *J. Chem. Soc., Chem. Commun.*, (1994), 775-776.

R. López, A. Fernández-Mayoralas. Enzymatic □-galactosidation of modified monosaccharides: Study of the enzyme selectivity for acceptor and its application to the synthesis of disaccharides. *J. Org. Chem.*, 59 (1994), 737-745.

R. López, E. Montero, F. Sánchez, J. Cañada y A. Fernández-Mayoralas. Regioselective acetylation of alkyl β-D-xylopyranosides by use of lipase PS in organic solvents and application to the chemoenzymatic synthesis of oligosaccharides. *J. Org. Chem.*, 59 (1994), 7027-7032.

J. M. Coterón, K. Singh, J. L. Asensio, M. Domínguez-Dalda, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas, J. Abad-Rodríguez y M. Nieto-Sampedro. Oligosaccharides structurally related to E-selectin ligands are inhibitors of neural cell division: Synthesis, conformational analysis, and biological activity. *J. Org. Chem.*, 60 (1995) 1502-1519.

B. Aguilera y A. Fernández-Mayoralas. Nucleophilic displacements on a cyclic sulphamidate derived from allosamine: Application to the synthesis of thio-oligosaccharides. *Chem. Commun.*, (1996) 127-128.

M. Nieto-Sampedro, C. Bailón, A. Fernández-Mayoralas, M. Martín-Lomas, B. Mellstrom y J. R. Naranjo. Experimental brain tumors: Growth arrest and destruction induced by a blood group-related tetrasaccharide. J. Neuropath. Exp. Neur., 55 (1996) 169-177.

J. J. Aragón, F. J. Cañada, A. Fernández-Mayoralas, R. López, M. Martín-Lomas, D. Villanueva. A direct enzymatic synthesis of β-D-galactopyranosyl-D-xylopyranosides and their use to evaluate rat intestinal lactase activity *in vivo*. *Carbohydr. Res.*, 290 (1996) 209-216.

A. Fernández-Mayoralas. Synthesis and modification of carbohydrates using glycosidases and lipases. *Top. Curr. Chem.*, 186 (1997) 1-20.

M. Carpintero, A. Fernández-Mayoralas, C. Jaramillo. Protecting group-directed diastereoselective samarium diiodide-prometed carbocyclization: Application to the synthesis of cyclitols. *J. Org. Chem.* 62 (1997) 1916-1917.

B. Aguilera, A. Fernández-Mayoralas, C. Jaramillo. Use of cyclic sulfamidates derived from D-allosamine in nucleophilic displacement: Scope and limitations. *Tetrahedron*, 53 (1997) 5863-5876.

N. Khiar, I. Alonso, N. Rodríguez, A. Fernández-Mayoralas, J. Jiménez-Barbero, O. Nieto, F. Cano, C. Foces-Foces, M. Martín-Lomas. Chemical and enzymatic diastereoselective cleavage of β-D-galactopyranosylsulfoxides. *Tetrahedron Lett.*, 38 (1997) 8267-8270

E. Montero, J. Alonso, F. J. Cañada, A. Fernández-Mayoralas, M. Martín-Lomas. Regioselectivity of the enzymatic transgalactosidation of D- and L-xylose catalysed by b-galactosidase. *Carbohydr. Res.*, 305 (1998) 383-391.

B. Aguilera, A. Fernández-Mayoralas. Synthesis of a thio-analogue of Lewis X by regioselective opening of cyclic sulfamidates. *J. Org. Chem.*, 63 (1998) 2719-2723.

B. Aguilera, J. Jiménez-Barbero, A. Fernández-Mayoralas. Conformational differences between Fuc(1-3)GlcNAc and its thioglycoside analogue. *Carbohydr. Res.*, 308 (1998) 19-27.

C. Jaramillo, G. Corrales, A. Fernández-Mayoralas. Glycosyl phenyl sulfoxides as a source of glycosyl carbanions: Stereoselective synthesis of C-fucosides. *Tetrahedron Lett.*, 39 (1998) 7783-7786.

B. Aguilera, L. Romero-Ramírez, J. Abad-Rodríguez, G. Corrales, M. Nieto-Sampedro, A. Fernández-Mayoralas. Novel disaccharide inhibitors of human glioma cell division. *J. Med. Chem.*, 41 (1998), 4599-4606.

A. Fernández-Mayoralas Enzymatic synthesis of lactose analogues using glycosidases. In "Carbohydrate mimics", VCH, Weinheim,1998, págs. 511-521 .

J. L. Asensio, F. J. Cañada, A. García, M. T. Murillo, A. Fernández-Mayoralas, B. A. Johns, J. Kozak, Z. Zhu, C. R. Johnson, J. Jiménez-Barbero. Conformational behaviour of aza-C-glycosides: Experimental

demonstration of the relative role of the exo-anomeric effect and 1,3-type interactions in controlling the conformation of regular glycosides. *J. Am. Chem. Soc.*, 121 (1999) 11318-11329.

M. Carpintero, C. Jaramillo, A. Fernández-Mayoralas. Stereoselective Synthesis of Carba- and C-Glycosyl Analogs of Fucopyranosides. *Eur. J. Org. Chem.*, (2000) 1285-1296.

C. Fernández, O. Nieto, J. A. Fontenla, E. Rivas, A. Fernández-Mayoralas. Synthesis and biological studies of glycosyl-dopamine derivatives as potential antiparkinsonian agents. *Carbohydr. Res.*, 327 (2000) 353-365.

J. M. Coterón, J. L. Chiara, A. Fernández-Mayoralas, J. M. Fiandor, N. Valle. Stereocontrolled glycosylation of sordaricin in the presence of ammonium salts. *Tetrahedron Lett.*, 41 (2000) 4373-4377.

J. M. Bueno, J. M. Coterón, J. L. Chiara, A. Fernández-Mayoralas, J. M. Fiandor, N. Valle. Stereoselective synthesis of the antifungal GM222712. *Tetrahedron Lett.*, 41 (2000) 4379-4382.

J. Abad-Rodríguez, M. Bernabé, L. Romero-Ramírez, M. Vallejo-Cremades, A. Fernández-Mayoralas, M. Nieto-Sampedro. Purification and structure of neurostatin, an inhibitor of astrocyte division of mammalian brain. *J. Neurochem.*, 74 (2000) 2547-2556.

G. Corrales, A. Fernández-Mayoralas, E. García-Junceda, Y. Rodríguez. A new strategy for liquid-phase synthesis of disaccharides based on the use of glycosidases. *Biocat. Biotrans.*,. 18 (2000) 271-281.

R. De Santiago, A. Fernández-Mayoralas, E. García-Junceda. Enzymatic synthesis of disaccharides by b-galactosidase-catalyzed glycosylation of a glycocluster. *J. Mol. Cat. B: Enzymatic*, 11 (2000), 71-79.

M. Carpintero, A. Fernández-Mayoralas, J. Jiménez-Barbero. The conformational behaviour of fucosyl and carbafucosyl mimetics in the free and in the protein bound state. *Eur. J. Org. Chem.*, (2001), 681-689.

M. Carpintero, I. Nieto, A. Fernández-Mayoralas. Stereoselective synthesis of a- and b-C-glycosides from glycosyl sulfoxides: Scope and limitations. *J. Org. Chem.,* 66 (2001) 1768-1774.

A. Bastida, A. Fernández-Mayoralas, R. Gómez, F. Iradier, J. C. Carretero, E. García-Junceda. Heterologous over-expression of a-1,6-fucosyltransferase from *Rhizobium* sp. Application to the synthesis of the trisaccharide β-D-GlcNAc(1-4)-(α-L-fuc-(1-6))-D-GlcNAc, study of the acceptor specificity, and evaluation pf polyhydroxylated indolizidenes as inhibitors. *Chem. Eur. J.*, 7 (2001) 2390-2397.

C. Alhambra, J. Castro, J. L. Chiara, E. Fernández, A. Fernández-Mayoralas, J. M. Fiandor, S. García-Ochoa, M. D. Martín-Ortega. An improved two-resin method for the cleavage of tertiary amines from REM resins. *Tetrahedron Lett.,* 42 (2001) 6675-6678.

M. Carpintero, A. Bastida, E. García-Junceda, J. Jiménez-Barbero, A. Fernández-Mayoralas. Synthesis of carba- and C-fucopyranosides an their evaluation as α-fucosidase inhibitors. Analysis of unusual conformation adopted by an amino-C-fucopyranoside. *Eur. J. Org. Chem.*, (2001) 4127-4135.

A. Bastida, A. Fernández-Mayoralas, E. García-Junceda. C- Terminal truncation of α-1,6-fucosyltransferase from *Rhizobium* sp. does not annul the transferase activity of the enzyme. *Bioorg. Med. Chem.*, 10 (2002) 737-742.

G. A. Abraham, A. Gallardo, J. San Román, G. Domenech, A. Fernández-Mayoralas, M. Zurita, J. Vaquero. Graft copolymers of poly(e-caprolactone) onto acrylic backbones as suitable polymer matrices for releasing a glycoside antitumoral drug. *J. Biomed. Mat. Res.,* 64A (2003) 638-647.

A. Fernández-Mayoralas, N. de la Figuera,. M. Zurita, J. Vaquero, G. A. Abraham, J. San Román, M. Nieto-Sampedro. Central neural tumor destruction by controlled release of a synthetic glycoside dispersed in a biodegradable polymeric matrix. *J. Med. Chem.*, 46 (2003) 1286-1288.

C. De Torres, A. Fernández-Mayoralas. Chemoenzymatic polymer-supported liquid phase synthesis of glucose g-aminobutiric acid. *Tetrahedron Lett.*, 44 (2003) 2383-2385.

C. Fernández, O. Nieto, J. A. Fontenla, E. Rivas, M. L. De Ceballos, A. Fernández-Mayoralas . Synthesis of glycosyl derivatives as dopamine prodrugs: Interaction with glucose carrier GLUT-1. *Org. Biomol. Chem.*, 1 (2003) 767-77.

C. De Torres, A. Fernández-Mayoralas . Lipase-catalyzed regioselctive acetylations and deacetylations of MeOPEG-bound xylopyranosides. *Lett. Org. Chem.*, 1 (2004) 99-101.

E. García-Junceda, J. F. García, A. Bastida, A. Fernández-Mayoralas. Enzymes in the synthesis of bioactive compounds: the prodigious decades. *Bioorg. Med. Chem.* 12 (2004) 1817-1834.

M. Nieto-Sampedro, E. Doncel, A. Fernández-Mayoralas. Natural, synthetic and semisynthetic glycolipid inhibitors of glioma growth. *Expert Opin. Ther. Pat,* 14 (2004) 487-497.

F. Calderón, M. Carpintero, E. García-Junceda, A. Fernández-Mayoralas, A.Bastida. Structure/activity relationship of carba and C-fucopyranosides as inhibitors of α-1,6-fucosyltransferases by molecular modelling and kinetic studies. *Lett. Org. Chem.*, 2 (2005) 247-254.

F. Calderón, R. Fernández, F. Sánchez, A. Fernández-Mayoralas. Asymmetric Aldol Reaction Using Immobilized Proline on Mesoporous Support. *Adv. Synth. Catal.*, 347 (2005) 1395-1403.

C. Hermida, G. Corrales, O. Martínez-Costa, A. Fernández-Mayoralas, J. J. Aragón. Non-invasive evaluation of intestinal lactase with 4-galactosylxylose: comparison with 3- and 2-galactosylxyloses and optimization of the method in rats. *Clin. Chem.*, 52 (2006) 270-277.

F. Calderón, E. G. Doyagüez, , A. Fernández-Mayoralas. Synthesis of azasugars through a proline-catalyzed reaction. *J. Org. Chem.*, 71 (2006) 6258-6261.

I. García-Álvarez, G. Corrales, E. Doncel-Pérez, A. Muñoz, M. Nieto-Sampedro, A. Fernández-Mayoralas. Design and Synthesis of Glycoside Inhibitors of Glioma and Melanoma Growth. *J. Med. Chem.*, 50 (2007) 364-373.

I. García-Álvarez, L. Garrido, A. Fernández-Mayoralas. Studies on uptake of glucose derivatives by red blood cells. *Chem MedChem.*, 2 (2007) 496-504.

F. Freire, F. Calderón, J. M. Seco, A. Fernández-Mayoralas, E. Quínoa, R. Riguera. Relative and absolute stereochemistry of secondary/secondary diols: low temperature ^{1}H NMR of their bis-MPA esters. *J. Org. Chem.*, 72 (2007) 2297-2301.

C. Hermida, G. Corrales, J. Cañada, J. J. Aragón, Alfonso Fernández-Mayoralas. Optimizing the enzymatic synthesis of β-D-galactopyranosyl-D-xyloses for their use in the evaluation of lactase activity in vivo. *Bioorg. Med. Chem.*, (2007), in press.

4. PATENTS

A. Fernández-Mayoralas, M. Martín-Lomas. Nuevos procedimientos de obtención de 3-metilactosa utilizable para la evaluación de la lactasa intestinal. Nº 539908. Year: 1985. Assignee:CSIC. Countries: Spain.

J. J. Aragón, G. Corrales, A. Fernández-Mayoralas, J. Jiménez-Barbero, M. Martín-Lomas, A. Rivera-Sagredo, D. Villanueva. Procedimiento de obtención de 4-O-β-D-galactopiranosil-D-xilosa utilizable para la evaluación diagnóstica de la lactasa intestinal. Nº 9001680. Year: 1990. Assignee: CSIC. Countries: Spain.

F. F. Santos Benito, M. Nieto Sampedro, A. Fernández-Mayoralas, M. Martín Lomas. Oligosaccharides used for inhibiting the mitosis of astrocytes and tumoral cells of the nervous system, and metods for obtaining them. Nº 9102522. Year: 1991. Assignee: CSIC. Countries: Europe and USA.

J. J. Aragón, F. J. Cañada, A. Fernández-Mayoralas, R. López, M. Martín-Lomas, y D. Villanueva. β-D-galactopyranosyl-D-xyloses used to evaluate the lactase activity, and enzymatic methods for obtaining them. la evaluación diagnóstica de la lactasa intestinal. Nº 9502185. Year: 1995
Assignee: CSIC-UAM. Countries: Europe and USA.

F. J. Cañada, A. Fernández-Mayoralas, M. Martín-Lomas, E. Montero. Mejoras en el procedimiento de obtención de β-D-galactopiranosil-xilosas utilizables para la evaluación diagnóstica de la lactasa intestinal. Nº: 9701156. Year: 1997. Assignee: CSIC-UAM. Countries: Spain.

C. Fernández, A. Fernández-Mayoralas, J. A. Fontenla, O. Nieto, E. Rivas. Derivados de glicosil-diacil dopamina y sus sales como agentes potenciales de reposición de dopamina en cerebro y su

procedimiento de obtención. Nº: 9902827. Year: 1999. Assignee: CSIC-Univ. Santiago de Compostela. Countries: Spain.

E. García-Junceda, A. Fernández-Mayoralas, A. Bastida. Dominio soluble con actividad enzimática a 1,6-fucosiltransferasa de Rhizobium sp. Nº: 200001386. Year: 2000. Assignee: CSIC. Countries: Spain.

M. Bernabé, A. Fernández-Mayoralas, M. Nieto, J. Vaquero, M. Zurita. Glicósidos de N-acetil-6-O-[2,2-bis(hidroximetil)-3-hidroxipropil]-D-glucosamina, procedimiento de obtención y uso en el tratamiento de tumores cerebrales. Nº: 200000982 (PCT: ES01/00138). Year: 2000. Assignee: CSIC-Clínica Puerta de Hierro. Countries: Europe.

F. J. Cañada, G. Corrales, A. Fernández-Mayoralas, M. Martín-Lomas, Juan José Aragón. Un procedimiento enzimático para obtener 4-O-β-D-galactopiranosil-D-xilosa, 4-O-β-D-galactopiranosil-D-xilosa obtenida de acuerdo con el procedimiento, composiciones que la contienen y su uso en la evaluación de la lactasa intestinal. Nº: 200101419. Year: 2001. Assignee: CSIC-UAM. Countries: Spain.

Exhibit B

Combinatorial carbohydrate chemistry

Lisa A Marcaurelle and Peter H Seeberger*

The application of combinatorial chemistry to the synthesis of carbohydrate-based compound collections has received increased attention in recent years. New strategies for the solution-phase synthesis of oligosaccharide libraries have been reported, and the use of monosaccharides as scaffolds in the generation of combinatorial libraries has been described. Novel approaches to the assembly of carbohydrate-based antibiotics, such as aminoglycoside analogs and vancomycin derivatives, have also been disclosed.

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Current Opinion in Chemical Biology 2002, 6:289–296

1367-5931/02/$ – see front matter

Published online 20 March 2002

Abbrevation
RGD Arg–Gly–Asp

Introduction

Combinatorial chemistry has become an important tool in modern drug development. Although carbohydrate-based compounds hold great potential as therapeutic agents, the application of combinatorial chemistry to this class of biomolecules has only recently elicited attention. The challenges associated with carbohydrate synthesis, including laborious protecting group manipulations and the need for regioselective and stereoselective glycosylation reactions, are primarily responsible for the lack of more intense efforts. The high degree of functionalization and diverse stereochemistry of carbohydrates, the very properties that render them attractive members of compound libraries, are responsible for the complications encountered by the experimentalist. In addressing and overcoming these challenges, the synthesis of a number of carbohydrate-based libraries has been achieved. This review highlights recent progress in the combinatorial synthesis of carbohydrates, including the development of new carbohydrate-based antibiotics and the use of carbohydrates as scaffolds for the synthesis of stereodiverse libraries. Recent advancements in solid-phase oligosaccharide synthesis and its application to carbohydrate libraries is also discussed.

Several excellent articles reviewing combinatorial carbohydrate synthesis have appeared prior to 2000 [1•,2,3]. This article focuses primarily on strategies reported in the past two years. The synthesis of glycopeptide libraries and related glycoconjugates has been reviewed recently and thus will not be covered [4••,5].

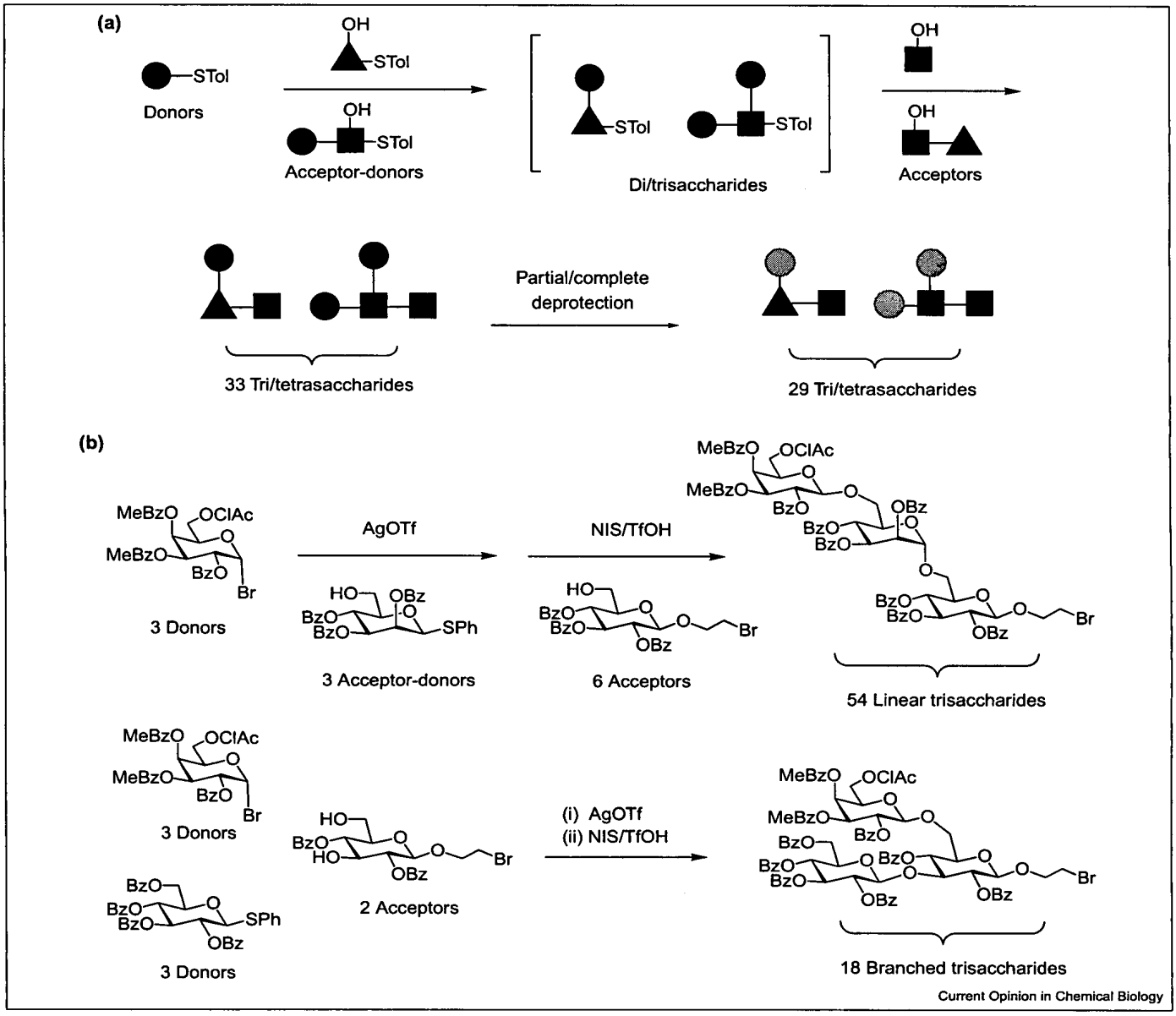
Combinatorial oligosaccharide libraries

In the past five years, the combinatorial synthesis of oligosaccharide libraries has been carried out both in solution and on solid support [1•,2,3]. During the period covered by this review, only two approaches to the combinatorial synthesis of oligosaccharides have been reported [6••,7•]. Both reports describe similar solution-phase approaches, employing sequential one-pot glycosylation strategies. Ye and Wong [6••] made use of their programmable one-pot glycosylation technology [8], which has been employed for the synthesis of a number of structures, including the tumor-associated hexasaccharide Globo-H [9•]. With the aid of anomeric reactivity values determined with the computer program OptiMer™, the construction of a small library of trisaccharides and tetrasaccharides was accomplished using a panel of monosaccharide and disaccharide donors. The sequential reaction of thioglycosides of varying reactivity produced a library of 33 oligosaccharides, which were partially or completely deprotected to create 29 additional compounds (Figure 1a).

In the second approach, Takahashi *et al.* [7•] reported the rapid assembly of a library of linear and branched trisaccharides by using a combination of donors, including glycosyl bromides, thioglycosides and 2-bromoethyl glycosides (Figure 1b). Selective activation of the bromide and thioglycoside donors with AgOTf and NIS/TfOH, respectively, enabled the generation of a library of 72 trisaccharides by sequential one-pot reactions on a manual synthesizer. It should be noted that each member of the library contains two sites for further elaboration. The chloroacetate group can be selectively removed for attachment of the trisaccharide to solid-support, while the bromoethyl glycoside can be modified by alkylation for the introduction of diversity at the anomeric position.

The synthesis of oligosaccharide libraries in solution has been quite fruitful. Still, the use of solid-phase methods for the construction of glycosidic linkages is attractive, because an excess of reagents may be used to ensure high yields and the number of purification steps is reduced. The solid-phase synthesis of oligosaccharide libraries was first reported by Kahne and co-workers [10] and later by Zhu and Boons [11]. Although no new methods for the solid-phase synthesis of oligosaccharide libraries have been reported during the past two years, a number of strategies for the solid-phase synthesis of oligosaccharides in general have been reported [12–14,15••,16•], including the automation of oligosaccharide assembly. The first automated solid-phase oligosaccharide synthesizer [15••] was used to prepare structures as large as branched dodecamers in less than one day. The synthesis was achieved using a re-engineered peptide synthesizer containing a coolable reaction vessel, utilizing glycosyl phosphates and glycosyl trichloroacetimidate building blocks (Figure 2). Each cycle involved the coupling of a building block to a growing resin-bound oligosaccharide and the removal of a protecting group to expose a single hydroxyl

Figure 1



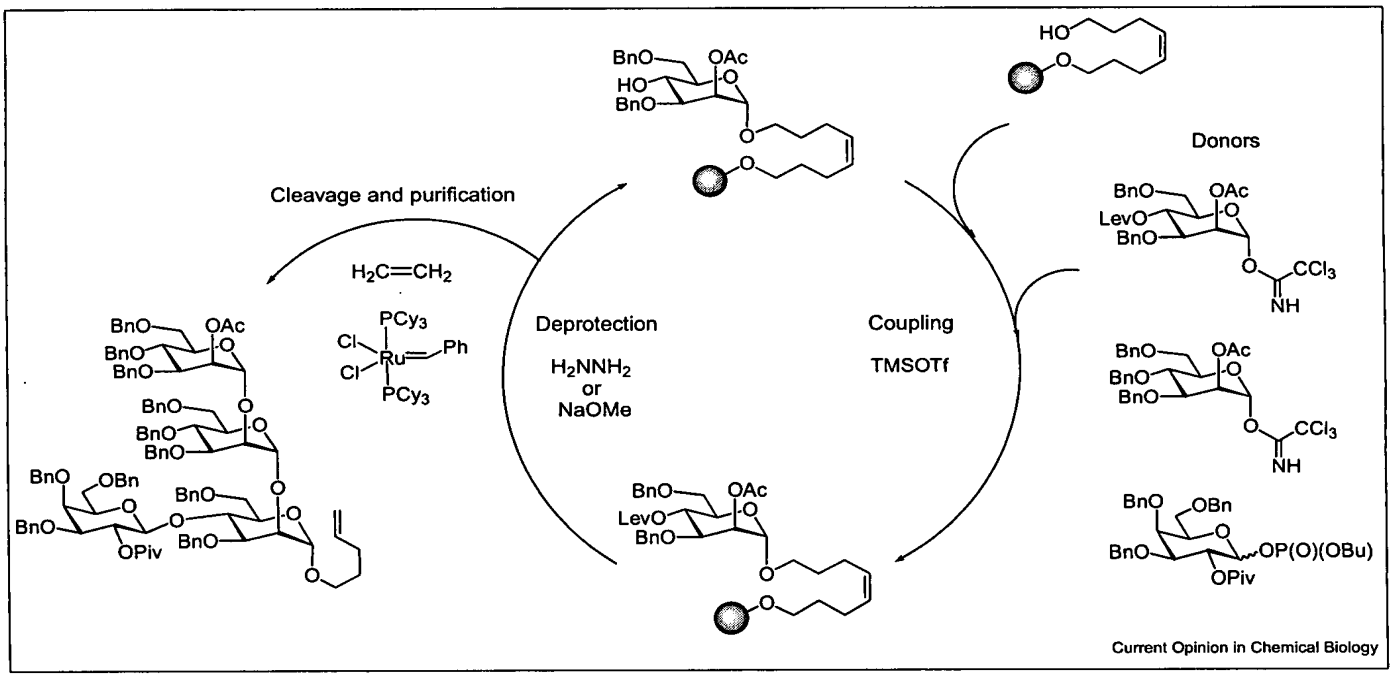
Novel approaches to oligosaccaharide libraries. **(a)** Wong's approach to the one-pot assembly of a library of linear and branched trisaccharides and tetrasaccharides. The sequential reaction of thioglycoside donors of varying reactivity produced a library of 33 oligosaccharides, which were partially or completely deprotected to afford 29 more compounds. **(b)** Takahashi's one-pot sequential assembly of a library of trisaccharides. Selective activation of glycosyl bromide and thioglycoside donors with AgOTf and NIS/TfOH, respectively, yielded a library of 72 linear and branched trisaccharides. Bz, benzoyl group; NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid.

group for attachment of the next carbohydrate. A metathesis-cleavable octenediol linker enabled release of the oligosaccharide from the support using Grubb's catalyst. This method has recently been applied to the synthesis of a branched tetrasaccharide (Figure 2) corresponding to a portion of the cell-surface lipophosphoglycan of *Leishmania* parasites [16•]. Branching of the tetrasaccharide was achieved through the selective removal of different ester protecting groups. The automation of oligosaccharide synthesis is expected to greatly facilitate preparation of oligosaccharide libraries by parallel synthesis.

Carbohydrate scaffolds for combinatorial synthesis

Monosaccharides are particularly attractive scaffolds for the synthesis of combinatorial libraries. They are readily available, conformationally rigid, chiral and highly functionalized molecules, containing up to five hydroxyl

Figure 2



Solid-phase synthesis of a branched tetrasaccharide [16•] using an automated oligosaccharide synthesizer [15••]. Bn, benzyl group. Cy, cyclohexyl group; Lev, levulinoyl group; Piv, pivaloyl group.

groups for the introduction of a diverse range of side chains. A variety of synthetic routes to these scaffolds have been reported [17,18] since carbohydrates were first described as 'privileged platforms' [19,20].

Recently, a focused combinatorial library of 126 mimetics of the Arg–Gly–Asp (RGD) peptidic sequence based on a sugar scaffold was rationally designed aided by molecular modeling [21•]. Although carbohydrate scaffolds had previously served as peptidomimetics, this was the first report of a combinatorial library of this class of compounds. D-Xylose was selected as a scaffold for the introduction of acidic and basic functional groups at various positions in order to achieve a high degree of stereodiversity. The α- and β-allyl glycosides of D-xylose (Figure 3a) were modified by benzylation to yield a total of 14 compounds, composed of a mixture of mono-, di- and trihydroxy derivatives. Alkylation with *t*-butylbromoacetate fashioned the corresponding ester derivatives. Using a 'mix-and-split strategy', the 14 compounds were elaborated into a library of 126 members that were functionalized with various amines. Using this strategy, an RGD mimic was identified that displayed activity equal to a known peptide-based inhibitor (RGDS) of integrin-mediated adhesion. The active compound contained an α-linked *N*-propyl substituent and a carboxylic acid at position 4. This method is now being applied to the synthesis of libraries of other biologically relevant peptidomimetics.

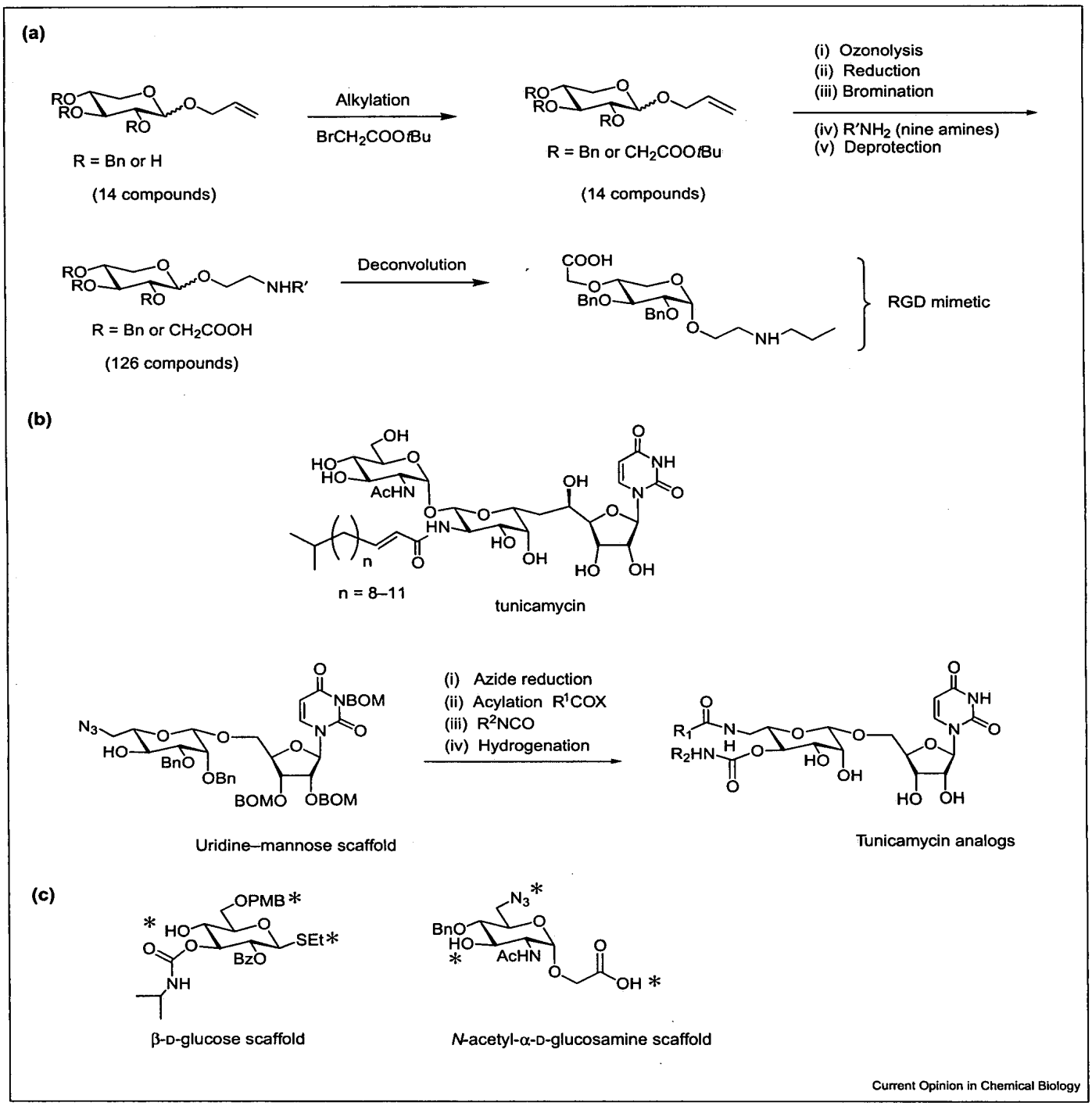
A second recently reported carbohydrate scaffold is based on tunicamycin (Figure 3b; [22]). Tunicamycins have been shown to inhibit a wide variety of lipid carrier-dependent protein glycosylations and are potential antibiotics, as they inhibit bacterial cell wall biosynthesis. Tunicamycins have not been used as therapeutics because they are toxic to mammalian cells, inhibiting all *N*-linked glycosylation. Analogs of tunicamycin may exhibit specific inhibitory effects towards eukaryotic and prokaryotic cells, potentially allowing for the targeting of pathogenic cells over mammalian cells. The tunicamycin scaffold incorporates two sites that can be derivatized orthogonally, an azide and a hydroxyl group (Figure 3b). Following azide reduction, modification of the disaccharide scaffold by acylation and amidation may generate a library of tunicamycin analogs.

Two additional scaffolds have been reported during the past two years (Figure 3c). The β-D-glucose [23] and *N*-acetyl-α-D-glucosamine [24] derived structures were synthesized for the purpose of generating carbohydrate-based libraries for broad screening and can be decorated at the positions indicated by asterisks in Figure 3c. The glucose scaffold is amenable to solid-support synthesis, resulting in an immobilized thioglycoside donor. Further diversity could be generated at the anomeric position by glycosylation.

Libraries of carbohydrate-based antibiotics

A number of antibiotics contain a glycan portion [25••]. Examples of carbohydrate-based antibiotics include

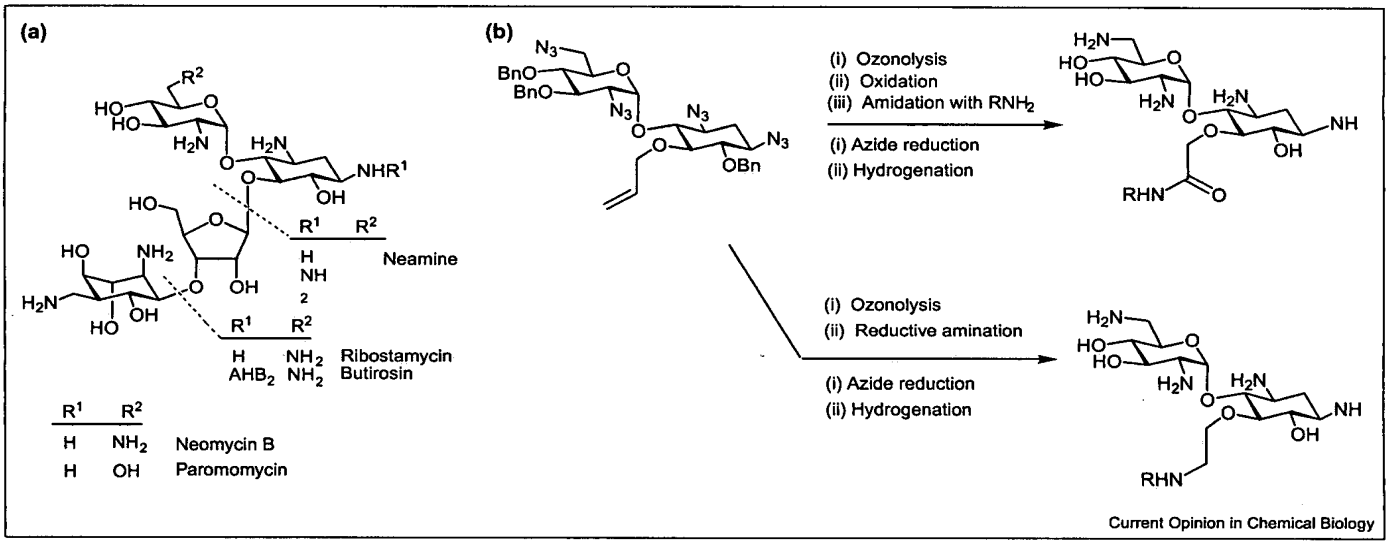
Figure 3



Use of carbohydrates as scaffolds for combinatorial synthesis. **(a)** Synthesis of a combinatorial library of peptidomimetics of the RGD sequence using D-xylose as a sugar scaffold [21•]. An RGD-mimic that was identified from the library is shown. **(b)** Structure of Sofia's carbohydrate scaffold based on tunicamycin containing two sites for functionalization. [22]. **(c)** Structure of carbohydrate scaffolds derived from β-D-glucose [23] and *N*-acetyl-α-D-glucosamine [24]. Sites for functionalization are indicated (*). BOM, benzyloxy methyl; PMB, *para*-methoxybenzyl group.

aminoglycosides, such as neomycin, kanamycin and streptomycin, and the glycopeptides vancomycin and teicoplanin. Because of the recent emergence of a number of drug-resistant bacterial strains, much effort has been focused on the generation of new structures with improved antibiotic activity. Wong and co-workers [26–28] have

Figure 4



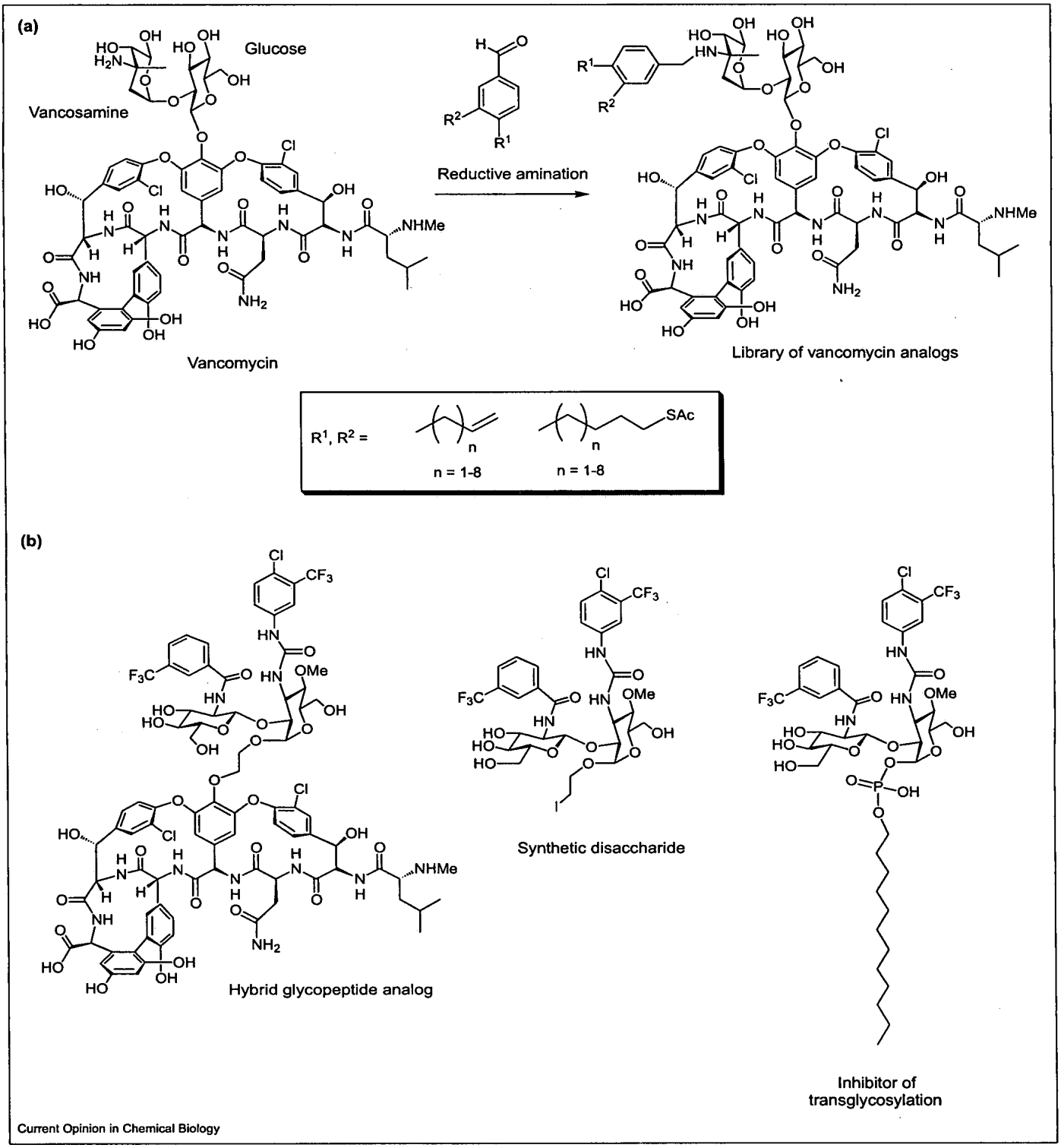
Synthesis of aminoglycoside libraries based on neamine assembled by reductive amination and amidation [29].

reported a number of library-based approaches for the discovery of new aminoglycoside antibiotics. Because of the size and complexity of aminoglycosides such as neomycin (Figure 4), a primary focus for the design of new antibiotics is the identification of simpler structures that retain the activity of the parent compound. Over the course of their studies, Wong *et al.* identified the naturally occurring pseudodisaccharide neamine as a core structure for the generation of new libraries of aminoglycoside mimetics [28]. A recent report described the synthesis of a library of neamine-based compounds and their RNA-binding properties [29]. The neamine library was constructed by reaction of the corresponding azide precursor with a variety of amines after conversion of the 5-*O*-allyl group to a reactive chemical handle (Figure 4). Amidation or reductive amination of the intermediate acid or aldehyde, followed by azide reduction and hydrogenation, yielded a library of compounds modified at the C-5 position of neamine.

The glycopeptide vancomycin (Figure 5a) has been used clinically for the past 40 years to treat infection by Gram-positive bacteria. The emergence of resistance to vancomycin in enterococcal strains has aroused considerable concern [30] and spurred vigorous efforts to develop novel antibiotics to combat these strains. In a series of recent reports, Nicolaou and co-workers [31,32,33••] described the construction of several libraries of vancomycin analogs, modified within the carbohydrate portion of the glycopeptide. Initial efforts were directed towards the replacement of the naturally occurring disaccharide with a panel of synthetic monosaccharides [31]. The glycosylation was performed on solid-phase using trichloracetimidate donors, with the aglycone attached to the resin by a new selenium-based safety-catch linker [32]. The monosaccharide analogs proved to be less active than the parent vancomycin against all bacterial strains. Having established the importance of the vancosamine moiety for antibacterial activity Nicolaou and co-workers turned their attention to the modification of the existing glycan by reductive amination. Reaction of vancomycin with a variety of substituted benzaldehydes (containing terminal alkenes or thioacetates) yielded a library of vancomycin analogs (Figure 5a). Biological evaluation of this library revealed several highly potent compounds effective against vancomycin-resistant strains. Dimerization of these compounds by disulfide formation and olefin metathesis led to the identification of an additional set of highly potent antibiotics [33••]. In this case, the discovery of active compounds was facilitated through the use of target-accelerated combinatorial synthesis (or dynamic combinatorial synthesis) [34,35].

It has been suggested that glycolipid derivatives of vancomycin (i.e. compounds containing a lipid-functionalized disaccharide) are active against resistant strains of bacteria because of their ability to inhibit the transglycosylation step of peptidoglycan biosynthesis [36,37]. If this model is correct, it should be possible to improve the activity of vancomycin derivatives by optimizing the glycolipid moiety for inhibition of transglycosylation. In order to test this hypothesis, Kahne and co-workers [38] devised a strategy for the synthesis of a new class of vancomycin analogs, termed hybrid glycopeptide antibiotics. To illustrate their approach, the aglycone was modified by alkylation with a synthetic disaccharide, corresponding to an analog of the known transglycosylase inhibitor moenomycin (Figure 5b). This disaccharide had been identified from a combinatorial library of moenomycin analogs [39]. The resulting hybrid

Figure 5



Strategies for the synthesis of vancomycin analogs. **(a)** Nicolaou's synthesis of vancomycin analogs by reductive amination with benzaldehyde derivatives, containing terminal alkenes and thioacetates [31]. Dimerization of the vancomycin analogs (by disulfide formation or olefin metathesis) led to the identification of potent antibiotics with activity against resistant bacterial strains [33••]. **(b)** Kahne's hybrid glycopeptide antibiotic, containing a disaccharide analog of the transglycosylase inhibitor moenomycin [38].

molecule, which contains the vancomycin aglycone in place of the lipid moiety, exhibits antibiotic activity far exceeding that of the individual components. This approach should greatly facilitate the synthesis of a large collection of vancomycin analogs, because the synthetically challenging glycosidic linkage is replaced with a simple ethylene glycol linker.

Conclusions

In light of the biological importance of oligosaccharides [40], the development of new strategies for their preparation is key to the advancement of our understanding of various carbohydrate–protein interactions and the discovery of new therapeutic agents. The application of combinatorial synthesis to the production of carbohydrate-based libraries has received increased attention in recent years. Combinatorial strategies have been applied to the discovery of new carbohydrate-based antibiotics, including derivatives of vancomycin [31,33••,38] and aminoglycosides [29], and novel one-pot glycosylation strategies have been employed for the generation of oligosaccharide libraries [6••,7•]. Recent advances in solid-phase oligosaccharide synthesis, resulting in the development of an automated synthesizer [15••], are expected to facilitate future progress in the assembly of carbohydrate-based libraries.

Acknowledgements

LAM would like to thank the NIH for a post-doctoral fellowship.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Schweizer F, Hindsgaul O: **Combinatorial synthesis of carbohydrates.** *Curr Opin Chem Biol* 1999, 3:291-298.

• This is a good review of the literature prior to 1999 on the combinatorial synthesis of carbohydrates, including the preparation of libraries of oligosaccharides and glycomimetics, and the use of carbohydrates as scaffolds.

2. Sofia MJ: **Carbohydrate-based combinatorial libraries.** *Mol Diversity* 1998, 3:75-94.

3. Kahne D: **Combinatorial approaches to carbohydrates.** *Curr Opin Chem Biol* 1997, 1:130-135.

4. St Hilaire PM, Meldal M: **Glycopeptide and oligosaccharide libraries.** *Angew Chem Int Ed Engl* 2000, 39:1162-1179.

•• This is an excellent review on the synthesis of glycopeptide and oligosaccharide libraries, including the screening of carbohydrate–protein interactions.

5. Barkley A, Arya P: **Combinatorial chemistry toward understanding the function(s) of carbohydrates and carbohydrate conjugates.** *Chemistry* 2001, 7:555-563.

6. Ye X-S, Wong C-H: **Anomeric reactivity-based on one-pot oligosaccharide synthesis: a rapid route to oligosaccharide libraries.** *J Org Chem* 2000, 65:2410-2431.

•• The OptiMer™ strategy was applied to the rapid assembly of a library of trisaccharides and tetrasaccharides. This was one of the first oligosaccharide libraries created by a one-pot approach.

7. Takahashi T, Adachi M, Matsuda A, Doi T: **Combinatorial synthesis of trisaccharides via solution-phase one-pot glycosylation.** *Tetrahedron Lett* 2000, 41:2599-2603.

• A library of 72 trisaccharides was constructed from a set of different glycosyl donors and selective activators. The solution-phase assembly of the library was achieved using a manual synthesizer.

8. Zhang Z, Ollman IR, Ye X-S, Wischnat R, Baasove T, Wong C-H: **Programmable one-pot oligosaccharide synthesis.** *J Am Chem Soc* 1999, 121:734-755.

9. Burkhart F, Zhang ZY, Wacowich-Sgarbi S, Wong CH: **Synthesis of the Globo-H hexasaccharide using the programmable reactivity-based one-pot strategy.** *Angew Chem Int Ed Engl* 2001, 40:1274-1277.

• The potential of the OptiMer™ strategy for synthesis planning was demonstrated with the one-pot synthesis of the tumor-associated carbohydrate antigen Globo-H.

10. Liang R, Yan L, Loebach J, Ge M, Uozumi Y, Sekanina K, Horan N, Gildersleeve J, Thompson C, Smith AB *et al.*: **Parallel synthesis and screening of a solid phase carbohydrate library.** *Science* 1996, 274:1520-1522.

11. Zhu T, Boons G-J: **A two-directional approach for the solid-phase synthesis of trisaccharide libraries.** *Angew Chem Int Ed Engl* 1998, 37:1898-1900.

12. Zhu T, Boons G-J: **A novel and efficient synthesis of a dimeric Le(x) oligosaccharide on polymeric support.** *J Am Chem Soc* 2000, 122:10222-10223.

13. Knerr L, Schmidt R: **Solid-phase synthesis of a branched hexasaccharide related to the lacto-*N*-hexaose.** *Eur J Org Chem* 2000:2803-2808.

14. Roussell F, Knerr L, Schmidt R: **Solid-phase synthesis of lactose-containing oligosaccharides.** *Eur J Org Chem* 2001:2067-2073.

15. Plante OJ, Palmacci ER, Seeberger PH: **Automated solid-phase synthesis of oligosaccharides.** *Science* 2001, 291:1523-1527.

•• The authors describe the first automated solid-phase oligosaccharide synthesizer for the assembly of linear and branched structures up to dodecasaccharides. This synthesizer may eventually facilitate the rapid parallel preparation of oligosaccharide libraries.

16. Hewitt MC, Seeberger PH: **Automated solid-phase synthesis of a branched *Leishmania* cap tetrasaccharide.** *Org Lett* 2001, 3:3699-3702.

• This automated synthesis of a branched tetrasaccharide was carried out on the solid-phase oligosaccharide synthesizer. Branching of the oligosaccharide was achieved through the selective removal of different ester-protecting groups.

17. Wunberg T, Kallus C, Opatz T, Henke S, Schmidt W, Kunz H: **Carbohydrates as multifunctional chiral scaffolds in combinatorial synthesis.** *Angew Chem Int Ed Engl* 1998, 37:2503-2505.

18. Sofia MJ, Hunter R, Chan TY, Vaughan A, Dulina R, Wang H, Gange D: **Carbohydrate-based small-molecule scaffolds for the construction of universal pharmacophore mapping libraries.** *J Org Chem* 1998, 63:2802-2803.

19. Hirschmann R, Nicolaou KC, Pietranico S, Leahy EM, Salvino J, Arison B, Cichy MA, Spoors PG, Shakespeare WC, Sprengeler PA *et al.*: ***De novo* design and synthesis of somatostatin non-peptide peptidomimetics utilizing β-D-glucose as a novel scaffolding.** *J Am Chem Soc* 1993, 115:12550-12568.

20. Hirschmann R, Hynes JJ, Cichy-Knight MA, van Rijn RD, Sprengeler PA, Spoors PG, Shakespeare WC, Pietranico-Cole S, Barbosa J, Liu J *et al.*: **Modulation of receptor and receptor subtype affinities using diastereomeric and enantiomeric monosaccharide scaffolds as a means to structural and biological diversity. A new route to ether synthesis.** *J Med Chem* 1998, 41:1382-1391.

21. Moitessier N, Dufour S, Chretien F, Thiery JP, Maigret B, Chapleur Y: **Design, synthesis and preliminary biological evaluation of a focused combinatorial library of stereodiverse carbohydrate-scaffold-based peptidomimetics.** *Bioorg Med Chem* 2001, 9:511-523.

• This was the first report of a library of peptidomimetics constructed from a carbohydrate scaffold.

22. Silva DJ, Sofia MJ: **Novel carbohydrate scaffolds. Assembly of a uridine-mannose scaffold based on tunicamycin.** *Tetrahedron Lett* 2000, 41:855-858.

23. Hirschmann R, Ducry L, Smith AB III: **Development of an efficient, regio- and stereoselective route to libraries based on the beta-D-glucose scaffold.** *J Org Chem* 2000, 65:8307-8316.

24. Ghosh M, Dulina RG, Kakarla R, Sofia MJ: **Efficient synthesis of a stereochemically defined carbohydrate scaffold: carboxymethyl 2-acetamido-6-azido-4-*O*-benzyl-2-deoxy-alpha-D-glucopyranoside.** *J Org Chem* 2000, 65:8387-8390.

25. Ritter TK, Wong CH: **Carbohydrate-based antibiotics: a new approach to tackling the problem of resistance.** *Angew Chem Int Ed Engl* 2001, **40**:3508-3533.
•• This is an excellent review on the identification of new targets and strategies for antibiotic discovery, including the development of small-molecules as antibiotics to target carbohydrate receptors or carbohydrate-modifying enzymes. The use of modified aminoglycosides and glycopeptides as new antibiotics is discussed.

26. Park WKC, Auer M, Jaksche H, Wong CH: **Rapid combinatorial synthesis of aminoglycoside antibiotic mimetics: use of a polyethylene glycol-linked amine and a neamine-derived aldehyde in multiple component condensation as a strategy for the discovery of new inhibitors of the HIV RNA Rev responsive element.** *J Am Chem Soc* 1996, **118**:10150-10155.

27. Wong CH, Hendrix M, Manning DM, Rosenbohm C, Greenberg WA: **A library approach to the discovery of small molecules that recognize RNA: use of a 1,3-hydroxyamine motif as core.** *J Am Chem Soc* 1998, **120**:8319-8327.

28. Greenberg WA, Priestly ES, Sears PS, Alper PB, Rosenbohm C, Hendrix M, Hung S-C, Wong CH: **Design and synthesis of new aminoglycoside antibiotics containing neamine as an optimal core structure: correlation of antibiotic activity with *in vitro* inhibition of translation.** *J Am Chem Soc* 1999, **121**:6527-6541.

29. Sucheck SJ, Greenberg WA, Tolbert TJ, Wong CH: **Design of small molecules that recognize RNA: development of aminoglycosides as potential antitumor agents that target oncogenic RNA sequences.** *Angew Chem Int Ed Engl* 2000, **39**:1080-1083.

30. Walsh C: **Molecular mechanisms that confer antibacterial drug resistance.** *Nature* 2000, **406**:775-781.

31. Nicolaou KC, Cho SY, Hughes R, Winssinger N, Smethurst C, Labischinski H, Endermann R: **Solid- and solution-phase synthesis of vancomycin and vancomycin analogues with activity against vancomycin-resistant bacteria.** *Chemistry* 2001, **7**:3798-3823.

32. Nicolaou KC, Winssinger N, Hughes R, Smethurst C, Cho SY: **New selenium-based safety catch linkers: solid-phase semisynthesis of vancomycin.** *Angew Chem Int Ed Engl* 2000, **39**:1084-1088.

33. Nicolaou KC, Hughes R, Cho SY, Winssinger N, Labischinski H, Endermann R: **Synthesis and biological evaluation of vancomycin dimers with potent activity against vancomycin-resistant bacteria: target accelerated combinatorial synthesis.** *Chemistry* 2001, **7**:3824-3843.
•• The dimerization of vancomycin analogs was achieved by disulfide formation and olefin metathesis to give a number of highly potent antibiotics with activity against vancomycin-resistant bacterial strains. Combinatorial synthesis of the dimer libraries was carried out in the presence of vancomycin's target Ac_2–L-Lys–D-Ala–D-Ala.

34. Ganesan A: **Strategies for the dynamic integration of combinatorial synthesis and screening.** *Angew Chem Int Ed Engl* 1998, **37**:2828-2831.

35. Lehn J-M: **Dynamic combinatorial chemistry and virtual combinatorial libraries.** *Chemistry* 1999, **5**:2455-2463.

36. Ge M, Chen Z, Onishi HR, Kohler J, Silver LL, Kerns R, Fukuzawa S, Thompson C, Kahne D: **Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala.** *Science* 1999, **284**:507-511.

37. Kerns R, Dong SD, Fukuzawa S, Carbeck J, Kohler J, Silver LL, Kahne D: **The role of hydrophobic substituents in the biological activity of glycopeptide antibiotics.** *J Am Chem Soc* 2000, **122**:12608-12609.

38. Sun B, Chen Z, Eggert US, Shaw SJ, LaTour JV, Kahne D: **Hybrid glycopeptide antibiotics.** *J Am Chem Soc* 2001, **123**:12722-12723.

39. Sofia MJ, Allanson N, Hatzenbuhler NT, Jain R, Kakarla R, Kogan N, Liang R, Liu D, Silva DJ, Wang H *et al.*: **Discovery of novel disaccharide antibacterial agents using a combinatorial library approach.** *J Med Chem* 1999, **42**:3193-3198.

40. Varki A: **Biological roles of oligosaccharides: all of the theories are correct.** *Glycobiology* 1993, **3**:97-130.

Exhibit C

ELSEVIER

Carbohydrate diversity: synthesis of glycoconjugates and complex carbohydrates

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The fundamental role of glycoconjugates in many biological processes is now well appreciated and has intensified the development of innovative and improved synthetic strategies. All areas of synthetic methodology have seen major advances and many complex, highly branched carbohydrates and glycoproteins have been prepared using solution- and/or solid-phase approaches. The development of an automated oligosaccharide synthesizer provides rapid access to biologically relevant compounds. These chemical approaches help to produce sufficient quantities of defined oligosaccharides for biological studies. Synthetic chemistry also supports an improved understanding of glycobiology and will eventually result in the discovery of new therapeutics.

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Current Opinion in Biotechnology 2004, 15:615–622

This review comes from a themed issue on
Chemical biotechnology
Edited by Ronald Frank

Available online 22nd October 2004

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2004.10.001

Abbreviations

GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
PSA	prostate-specific antigen

Introduction

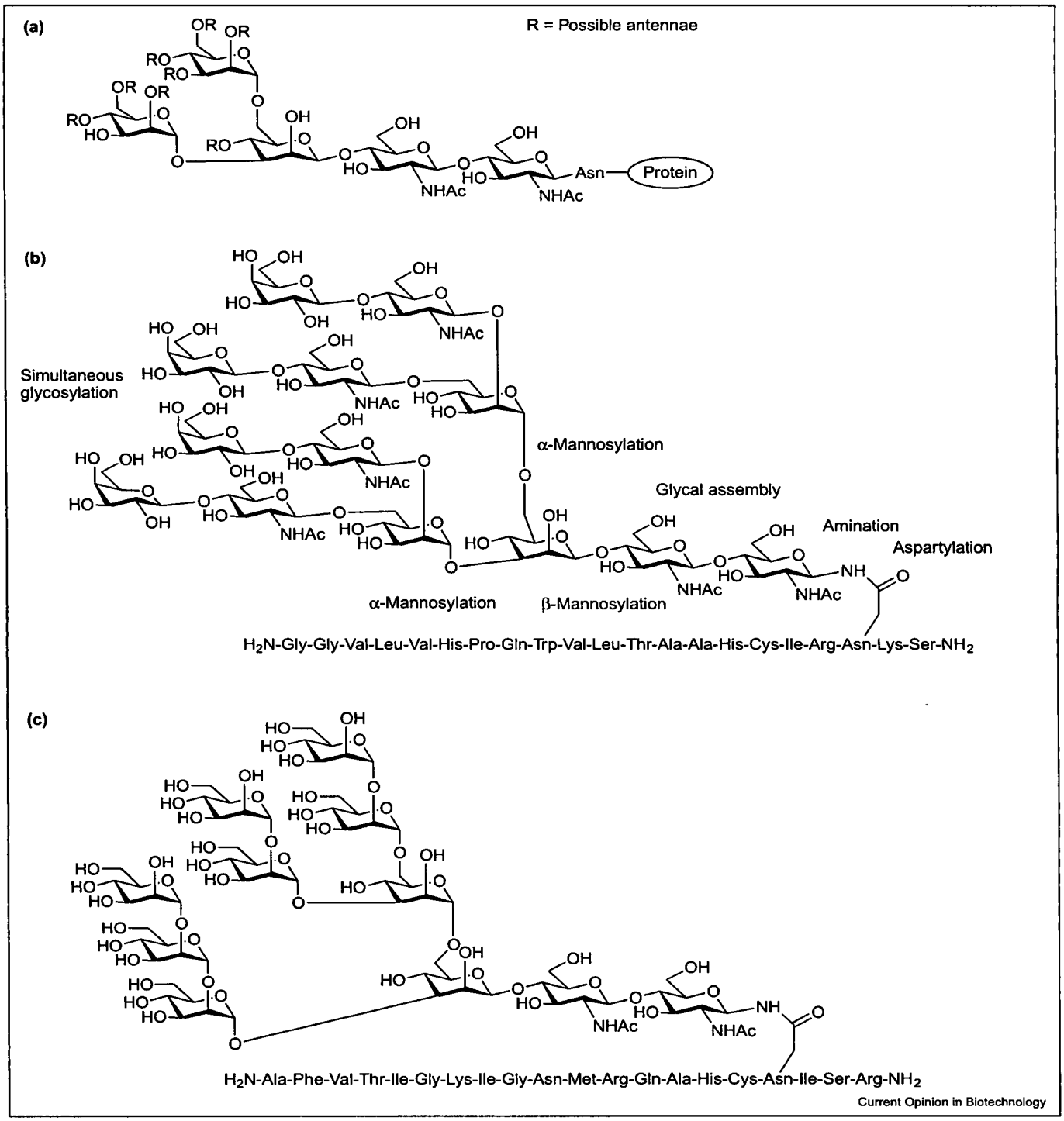
In addition to oligopeptides and oligonucleotides, oligosaccharides (glycans) constitute the third major class of naturally occurring biopolymers that play a fundamental role in many important biological processes. Glycans are commonly found in nature as glycoconjugates (glycoproteins or glycolipids) that show high structural diversity, greatly exceeding the diversity of proteins and nucleic acids.

In contrast to linear oligopeptides and oligonucleotides, oligosaccharides are often complex branched molecules and the glycan core is commonly attached to proteins and lipids. In nature, three major classes of glycans exist: *N*-linked glycans, *O*-linked glycans and glycosylphosphatidylinositol (GPI) anchors. Intensive research into the biological role of carbohydrates has led to an increased need for sufficient quantities of natural and modified glycoproteins; however, the isolation of carbohydrates from natural sources is extremely difficult owing to their structural complexity. Access to pure carbohydrates for biological, biochemical, biophysical and medicinal studies therefore relies on chemical and enzymatic synthesis [1,2]. Remarkable progress has been made in this area; however, further innovations are required to handle the structural complexity of oligosaccharides. Their preparation is technically difficult, extremely time-consuming and performed by a few specialized laboratories. The introduction of solid-phase synthesis strategies has significantly improved carbohydrate assembly, as an excess of reagent can be used to ensure high yields and to reduce the number of purification steps. The development of an automated oligosaccharide synthesizer [3•,4•,5••] has led to rapid access to complex carbohydrates of biological relevance. This review highlights recent advances in the synthesis of complex oligosaccharides and glycoproteins, primarily focusing on strategies published in the past two years.

N-Linked glycoproteins

N-Linked glycoproteins (*N*-glycans) are the most abundant in nature and are commonly divided into four groups: high-mannose, complex, hybrid and poly-*N*-acetyllactosamine glycans. Although the structural details are well established, little is known about their structure–activity relationship. In *N*-glycans, the oligosaccharide sidechain is attached to the protein via an asparagine amino acid. All *N*-glycans share the common pentasaccharide core structure (mannose)$_3$(*N*-acetylglucosamine)$_2$ (Man$_3$GlcNAc$_2$) shown in Figure 1a. Structural diversity is generated by variation in the substitution pattern of the pentasaccharide core, in the degree of branching and in the terminal sugars. The pentasaccharide core can be extended by up to five antennae. The preparation of the basic structure contains several synthetic challenges, including branching and the inclusion of a β-mannoside. Recently, two efficient partial syntheses of the core structure have been accomplished [6,7], selectively establishing the β-mannosidic linkage. The orthogonally protected β-mannosylated chitobiose trisaccharide with a terminal azido group serves as a key building block in the preparation of complex *N*-glycans. The entire pentasaccharide has been synthesized recently by Danishefsky and colleagues [8]

Figure 1



N-Linked glycoproteins. **(a)** Structure of the core pentasaccharide common to all *N*-glycans. The core structure can be extended by up to five antennae (R). **(b)** Structure of a prostate-specific antigen (PSA) glycopeptide. The crucial retrosynthetic steps of Danishefsky's [21•] strategy are shown. **(c)** Structure of the gp120 glycopeptide fragment, which is a possible target for an anti-HIV-vaccine. Protein sequences are shown using the three-letter amino acid code.

using Crich's β-mannosylation methodology [6] followed by a simultaneous di-α-mannosylation with a thiomannoside donor.

As an alternative to these solution-phase preparations, the synthesis of the core pentasaccharide selectively functionalized with one *N*-acetylglucosamine residue has been performed recently using a solid-phase approach [9]. The first automated solid-phase oligosaccharide synthesizer [5••] has been used to efficiently prepare the core pentasaccharide [10] by using an octenediol functionalized Merrifield's resin and three different building blocks: two monosaccharides and one disaccharide already containing the β-mannosidic linkage. Branching was achieved by simultaneous dimannosylation of the trisaccharide core.

Innovative synthetic methods have also provided access to more complex and highly branched *N*-glycans. Weiss and Unverzagt [11] have developed a general strategy for the preparation of multiantennary *N*-glycans. Crucial challenges in the synthesis of these sterically crowded bi- to tetra-antennary compounds is the sequence of introducing the building blocks and the steric demand of the building blocks. Complex biantennary *N*-glycans are also accessible via chemoenzymatic total synthesis. Elongation of synthetic oligosaccharides has been performed using glycosyltransferases to give full-length *N*-glycans [12,13].

Synthetic oligosaccharides are useful in gaining a more detailed understanding of glycoprotein quality control. In particular, maintenance of the integrity of protein folding has recently received significant attention. Ito and colleagues [14,15] accomplished a convergent and stereoselective route to the nonasaccharide $Man_8GlcNAc_2$ and the monoglucosylated dodecasaccharide α-$Glc_1Man_9GlcNAc_2$, a putative ligand of the molecular chaperones calnexin and calreticurin. These synthetic oligosaccharides might serve as molecular probes to detect glycoprotein–mannosidase-like protein recognition.

Glycoproteins are also important in the context of diagnostics, therapeutics and vaccines. The integration of oligosaccharides into glycoproteins is realized by converting them into anomeric glycosylamines, which is either performed by treatment with ammonium hydrogencarbonate or by reduction of anomeric glycosyl azides, and subsequent attachment to the peptide chain [16–18]. Guo and colleagues [19] attached a fucoslyated trisaccharide to the peptide of the CD52 antigen by using a solution-phase synthesis with solid-phase workup or a combined solution- and solid-phase approach. More complex oligosaccharides containing two thiol residues were linked to the same peptide by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis [20]. Because of their short peptide chain containing only 12 amino acids, their simple glycosylation pattern and their interesting bioactivity, these glycopeptides serve as useful models to study structure–activity relationships. The development of a universal strategy [21•] for the preparation of complex multibranched *N*-acetyllactosamine-type glycans from common precursors has led to the first chemical synthesis of normal and transformed prostate-specific antigen (PSA) glycopeptides (Figure 1b). PSA has been identified as a highly specific cancer marker that might enable the early diagnosis of prostate tumours.

N-Linked carbohydrates also play an important role in human immunodeficiency virus (HIV) retroviral pathogenesis. The HIV-1 surface envelope glycoprotein gp120 is highly glycosylated containing up to 24 *N*-linked high-mannose carbohydrates and shows biological functions in helper T-lymphocyte infections [22]. Seeberger and colleagues [23] developed a linear solution-phase synthesis of a triantennary high-mannose nonasaccharide from gp120 using just three monosaccharide building blocks. Employing a reactivity-based one-pot self-condensation approach, Wong and coworkers [24] prepared several high-mannose oligosaccharides, which efficiently inhibit the binding of the antibody 2G12 to gp120. More recently, Danishefsky and colleagues [25••,26••] described the first chemical synthesis of HIV gp120 fragments (Figure 1c), which serve as targets for an anti-HIV vaccine.

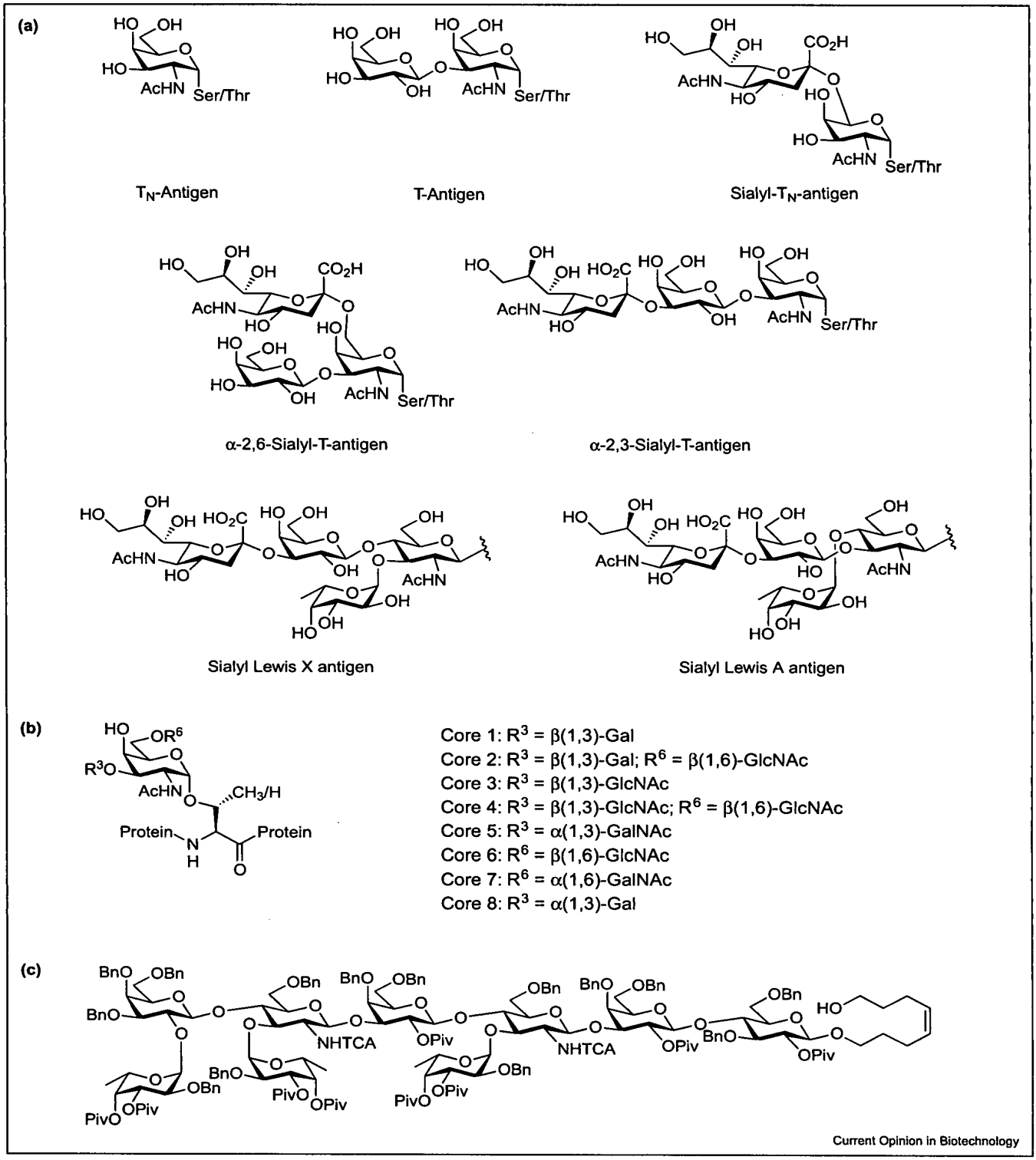
Many important glycoproteins are multiply glycosylated at fixed sites. Danishefsky's laboratory [27] recently disclosed a convergent method for the preparation of bifunctional glycopeptides: two glycopeptides are synthesized separately from their glycan and peptide precursors using standard procedures and subsequently coupled to yield the bifunctional compounds.

O-Linked glycoproteins

A second major group of biologically important glycoproteins are *O*-linked glycoproteins (*O*-glycans). The carbohydrate residue in *O*-glycans is covalently attached to the peptide backbone via the hydroxyl group of serine, threonine, tyrosine, hydroxyproline, hydroxylysine or another hydroxylated amino acid. In contrast to *N*-glycans, these glycoproteins show a higher degree of structural diversity and do not share a common core structure. Additional variety arises from further carbohydrate elongations of these backbones.

Tumour-associated antigens (Figure 2a) like the T_N-, T-, sialyl-T_N and sialyl-T antigens as well as the sialyl Lewis X and sialyl Lewis A antigens were first found in mucins. Mucins are a class of highly *O*-glycosylated proteins present on the surface of various types of epithelial cells. In normal tissue, the peptide backbone carries several complex oligosaccharides derived from the glycan core structures shown in Figure 2b, which are characterized by

Figure 2



O-Linked glycoproteins. **(a)** Structures of tumour-associated carbohydrate antigens that were first discovered in mucins. **(b)** Core structures of mucin-type *O*-linked glycans, a class of highly *O*-glycosylated proteins. **(c)** Structure of the Le^y-Le^x tumour marker. Ac, acetyl; Bn, benzyl; Piv, pivaloyl; TCA, trichloroacetyl.

an *N*-acetylgalactosamine unit α-*O*-linked to serine or threonine. An increased expression of mucins is usually prevalent in tumour cells, where the carbohydrate chains are modified due to incomplete glycosylation and premature sialylation. As tumour-associated glycans with peptide sequences of mucins constitute a promising target for the development of synthetic antitumour vaccines, the chemical synthesis of such glycoconjugates has received considerable attention and several reviews devoted to this field of research have been published [28–30,31•].

A solid-phase approach [32,33] has been used for the stereoselective construction of several different mucin-type *O*-glycans. Stepwise elongation of the carbohydrate led to the required highly glycosylated amino acid building blocks, which were then incorporated into a solid-phase glycopeptide synthesis. Other branched *O*-glycans have recently been prepared by an efficient one-pot glycosylation approach using either glycosyl fluoride [34] or thioglycoside [35] building blocks. As sialylated derivatives of tumour-associated antigens are also present on the surface of cancer cells, the preparation of *O*-linked sialyl oligosaccharides is important. Paulson and colleagues [36] demonstrated that recombinant sialyltransferases are ideal catalysts for the simple and efficient preparation of *O*-linked sialyl oligosaccharides by elongation of a synthetic glycosyl amino acid.

The application of non-natural amino acids in carbohydrate vaccines has also attracted considerable attention [37], as these unnatural linkages might give an increased immune response. Danishefsky's group [38] investigated the synthesis of different glycosyl hydroxynorleucines, each containing a tumour-associated carbohydrate antigen. While the glycosylation of trichloroacetimidate donors with the amino acid predominately afforded the corresponding α-*O*-linked product, the reaction with a glycal epoxide donor provided the β-*O*-linked product. The glycal methodology was also successfully applied to the synthesis of Lewis Y- and Globo-H-containing amino acids.

More recently, an automated synthesizer has been used to accelerate the synthesis of the Lewis^{y}-Lewis^{x} tumour marker (Figure 2c) and the Lewis X and Lewis Y blood group antigens [39••]. Only five monomers were necessary for the efficient construction of the three target structures.

GPI anchors

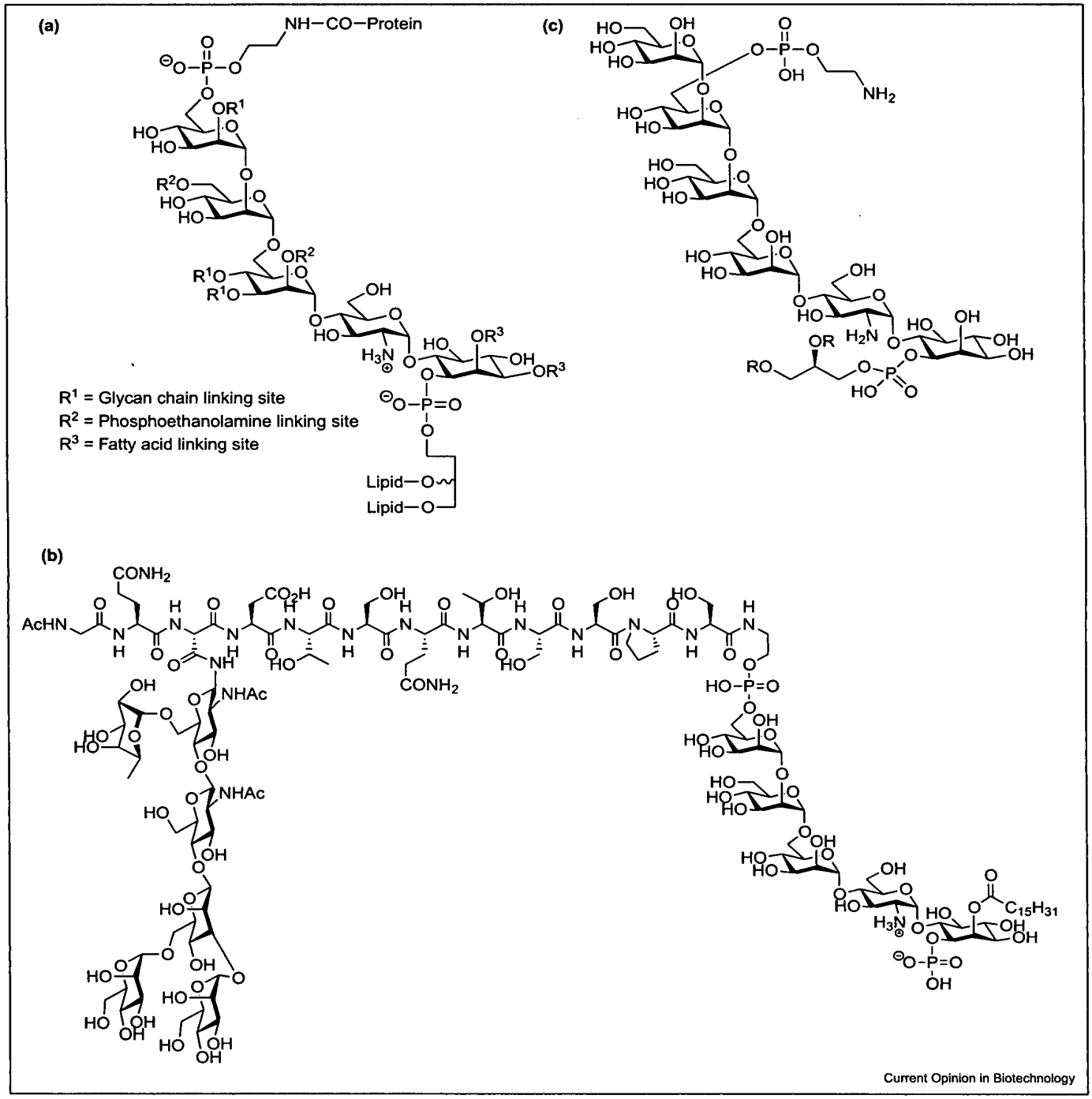
GPI-anchored proteins are involved in many biological and physiological processes and have attracted considerable attention since the first structure determination of a GPI in 1988 [40]. These naturally occurring glycolipids serve to attach proteins or glycoproteins onto eukaryotic cell membranes. All reported GPI structures share the basic core structure shown in Figure 3a with a linear tetrasaccharide attached to the 6-*O*-position of inositol. Besides this conserved general structure, considerable diversity exists within the GPI anchor family based on the variation of the substitution pattern on this pseudo-pentasaccharide backbone. In most cases, the core is further modified by species-specific carbohydrates, additional phosphoethanolamine units and variations in the lipid moiety. Proteins or glycoproteins are linked to the non-reducing end by their C termini or a phosphoethanolamine group. Owing to the structural complexity of the GPI anchors that requires a detailed knowledge of lipid, phosphate and oligosaccharide chemistry, many chemists have focused on the synthesis of these motifs [41•].

A linear solution-phase approach allows for the construction of complex GPI anchors and for the preparation of an orthogonally protected derivative of the phosphorylated pseudo-pentasaccharide core [42]. Another variable concept for the preparation of branched GPIs was developed by Pekari and Schmidt [43]. The efficiency of this approach was demonstrated by the synthesis of the GPI anchors of rat brain Thy-1 and scrapie prion protein in their water-soluble and lipidated forms. This approach also allows further attachment of peptide residues or biological markers to the GPI anchor. Reichardt and Martin-Lomas [44] reported a soluble support-based approach for the synthesis of the GPI backbone. This method, using a polyethylene glycol-grafted polystyrene resin functionalized with a Wang-chloride linker, can be applied to the preparation of a small library of GPI precursors.

CD52 antigens, simple GPI-anchored glycopeptides, are present on eukaryotic cells and play an important role in the human immune system. Initial studies aimed at the synthesis of sperm CD52, including the preparation of an acylated inositol [45] and the linkage to the peptide [46], were performed by Guo and colleagues. More recently, they reported [47••] the first synthesis of a skeleton structure of sperm CD52. In their strategy the glycopeptide and the GPI anchor were prepared separately and subsequently linked by an amide bond to give the glycopeptide–GPI conjugate (Figure 3b).

Synthetic GPIs are promising vaccine candidates against malaria, as shown in a mouse model [48]. Annually, malaria infects 5–10% of the world's population and kills about 3 million people each year. The malaria parasite *Plasmodium falciparum* expresses a large amount of GPI anchored to a protein, and the GPI structure (Figure 3c) has been identified as the malaria toxin. A solution-phase synthesis of two malaria vaccine candidates with a pseudo-hexasaccharide backbone has recently been reported by Seeberger and collagues [49]. This strategy allows for scale-up to procure compounds for preclinical

Figure 3



GPI anchors. **(a)** Basic core structure of all GPI anchors. **(b)** Skeleton structure of sperm CD52. **(c)** Structure of the malaria GPI vaccine candidate.

and clinical trials. The authors also demonstrated that the synthesis of this target can be automated effectively [50••]. The fully protected oligosaccharide was obtained in only 9 h starting from four monosaccharides and one disaccharide building block. Fraser-Reid and coworkers [51,52••] developed a method for the solution-phase

synthesis of a fully lipidated and phosphorylated malarial GPI pseudo-pentasaccharide using orthoesters and methyl α-D-glucopyranoside as the key building blocks.

Conclusions

Innovative synthetic methods are an important tool to create diverse carbohydrates. Recent advances in the preparation of complex oligosaccharides as well as entire glycoproteins containing *N*-glycans, *O*-glycans and GPI anchors have been highlighted in this review. Highly branched carbohydrates and biologically relevant oligosaccharides are now accessible via these methods, providing sufficient quantities for biological studies. The availability of defined synthetic glycoproteins and glycolipids will significantly support biological investigations. The development of new strategies for the preparation of carbohydrates is fundamental for the understanding of carbohydrate–protein interactions, biosynthetic pathways and structure–activity relationships and will allow for the discovery of new targets for therapeutics, diagnostics and vaccines. The introduction of an automated oligosaccharide synthesizer has greatly accelerated access to many highly branched carbohydrates, and a series of biologically relevant oligosaccharides has been efficiently prepared on this machine. Further improvement and extension of this technology could allow for the automated synthesis of complex glycoproteins, proteoglycans and glycolipids using only one instrument and will eventually enable even non-specialists to create biologically important compounds for biochemical, biophysical and medicinal applications.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Davis BG: **Synthesis of glycoproteins.** *Chem Rev* 2002, **102**:579-601.
2. Macmillan D, Daines AM: **Recent developments in the synthesis and discovery of oligosaccharides and glycoconjugates for the treatment of disease.** *Curr Med Chem* 2003, **10**:2733-2773.
3. • Seeberger PH: **Automated carbohydrate synthesis to drive chemical glycomics.** *Chem Commun* 2003:1115-1121.
   See annotation for [5••].
4. • Palmacci ER, Plante OJ, Hewitt MC, Seeberger PH: **Automated synthesis of oligosaccharides.** *Helv Chim Acta* 2003, **86**:3975-3990.
   See annotation for [5••].
5. •• Plante OJ, Palmacci ER, Seeberger PH: **Automated solid-phase synthesis of oligosaccharides.** *Science* 2001, **291**:1523-1527.
   The authors describe the first automated solid-phase oligosaccharide synthesizer for the preparation of linear and branched structures up to dodecasaccharides. New glycosylating agents, linker, coupling cycles and the use of high-resolution magic angle spinning NMR to follow reactions on polymer support are discussed.
6. Dudkin VY, Crich D: **A short synthesis of the trisaccharide building block of the *N*-linked glycans.** *Tetrahedron Lett* 2003, **44**:1787-1789.
7. Unverzagt C: **Synthesis of a core trisaccharide as a versatile building block for *N*-glycans and glycoconjugates.** *Chem Eur J* 2003, **9**:1369-1376.
8. Dudkin VY, Miller JS, Danishefsky SJ: **A concise route to the core pentasaccharide of *N*-linked glycoproteins.** *Tetrahedron Lett* 2003, **44**:1791-1793.
9. Wu XY, Grathwohl M, Schmidt RR: **Efficient solid-phase synthesis of a complex, branched *N*-glycan hexasaccharide: use of a novel linker and temporary-protecting-group pattern.** *Angew Chem Int Edit* 2002, **41**:4489-4493.
10. Ratner DM, Swanson ER, Seeberger PH: **Automated synthesis of a protected *N*-linked glycoprotein core pentasaccharide.** *Org Lett* 2003, **5**:4717-4720.
11. Weiss H, Unverzagt C: **Highly branched oligosaccharides: a general strategy for the synthesis of multiantennary *N*-glycans with a bisected motif.** *Angew Chem Int Edit* 2003, **42**:4261-4263.
12. Unverzagt C, Andre S, Seifert J, Kojima S, Fink C, Srikrishna G, Freeze H, Kayser K, Gabius HJ: **Structure-activity profiles of complex biantennary glycans with core fucosylation and with/without additional α2,3/α2,6 sialylation: synthesis of neoglycoproteins and their properties in lectin assays, cell binding, and organ uptake.** *J Med Chem* 2002, **45**:478-491.
13. Prahl I, Unverzagt C: **Enzymatic elongation of the LEC14 antigen generates a β-1,2 arm on N-glycans.** *Angew Chem Int Edit* 2002, **41**:4259-4262.
14. Matsuo I, Wada M, Manabe S, Yamaguchi Y, Otake K, Kato K, Ito Y: **Synthesis of monoglucosylated high-mannose-type dodecasaccharide, a putative ligand for molecular chaperone, calnexin, and calreticurin.** *J Am Chem Soc* 2003, **125**:3402-3403.
15. Matsuo I, Ito Y: **Synthesis of an octamannosyled glycan chain, the key oligosaccharide structure in ER-associated degradation.** *Carbohydr Res* 2003, **338**:2163-2168.
16. Totani K, Matsuo I, Ito Y: **Tight binding ligand approach to oligosaccharide-grafted protein.** *Bioorg Med Chem Lett* 2004, **14**:2285-2289.
17. Hojo H, Haginoya E, Matsumoto Y, Nakahara Y, Nabeshima K, Toole BP, Watanabe Y: **The first synthesis of peptide thioester carrying *N*-linked core pentasaccharide through modified Fmoc thioester preparation: synthesis of an *N*-glycosylated Ig domain of emmprin.** *Tetrahedron Lett* 2003, **44**:2961-2964.
18. Miller JS, Dudkin VY, Lyon GJ, Muir TW, Danishefsky SJ: **Toward fully synthetic *N*-linked glycoproteins.** *Angew Chem Int Edit* 2003, **42**:431-434.
19. Shao N, Xue J, Guo ZW: **Chemical synthesis of CD52 glycopeptides containing the acid-labile fucosyl linkage.** *J Org Chem* 2003, **68**:9003-9011.
20. Pratt MR, Bertozzi CR: **Chemoselective ligation applied to the synthesis of a biantennary N-linked glycoform of CD52.** *J Am Chem Soc* 2003, **125**:6149-6159.
21. • Dudkin VY, Miller JS, Danishefsky SJ: **Chemical synthesis of normal and transformed PSA glycopeptides.** *J Am Chem Soc* 2004, **126**:736-738.
    A universal method for the preparation of N-linked glycans from a common precursor has been established and efficiently applied to the synthesis of normal and transformed PSA fragments.
22. Bewley CA, Gustafson KR, Boyd MR, Covell DG, Bax A, Clore GM, Gronenborn AM: **Solution structure of cyanovirin-N, a potent HIV-inactivating protein.** *Nat Struct Biol* 1998, **5**:571-578.
23. Ratner DM, Plante OJ, Seeberger PH: **A linear synthesis of branched high-mannose oligosaccharides from the HIV-1 viral surface envelope glycoprotein gp120.** *Eur J Org Chem* 2002:826-833.
24. Lee HK, Scanlan CN, Huang CY, Chang AY, Calarese DA, Dwek RA, Rudd PM, Burton DR, Wilson IA, Wong CH: **Reactivity-based one-pot synthesis of oligomannoses: defining antigens recognized by 2G12, a broadly neutralizing anti-HIV-1 antibody.** *Angew Chem Int Edit* 2004, **43**:1000-1003.

25. Mandal M, Dudkin VY, Geng XD, Danishefsky S: **In pursuit of**
•• **carbohydrate-based HIV vaccines, Part 1: The total synthesis of hybrid-type gp120 fragments.** *Angew Chem Int Edit* 2004, **43**:2557-2561.

The first chemical synthesis of mature hybrid type HIV gp120 glycopeptide fragments is reported, which may serve as anti-HIV vaccines.

26. Geng XD, Dudkin VY, Mandal M, Danishefsky SJ: **In pursuit of**
•• **carbohydrate-based HIV vaccines, Part 2: The total synthesis of high-mannose-type gp120 fragments-evaluation of strategies directed to maximal convergence.** *Angew Chem Int Edit* 2004, **43**:2562-2565.

The first chemical synthesis of high-mannose type HIV gp120 glycopeptide fragments has been achieved using either a 'layered' or a 'block' assembly oligosaccharide approach. The glycans were then linked to gp120 peptide fragments by direct aspartylation.

27. Warren JD, Miller JS, Keding SJ, Danishefsky SJ: **Toward fully synthetic glycoproteins by ultimately convergent routes: a solution to a long-standing problem.** *J Am Chem Soc* 2004, **126**:6576-6578.

28. Dziadek S, Espinola CG, Kunz H: **Synthetic glycopeptides for the development of antitumour vaccines.** *Aust J Chem* 2003, **56**:519-543.

29. Dziadek S, Kunz H: **Synthesis of tumor-associated glycopeptide antigens for the development of tumor-selective vaccines.** *Chem Rec* 2004, **3**:308-321.

30. Marcaurelle LA, Bertozzi CR: **Recent advances in the chemical synthesis of mucin-like glycoproteins.** *Glycobiology* 2002, **12**:69R-77R.

31. Brocke C, Kunz H: **Synthesis of tumor-associated glycopeptide**
• **antigens.** *Bioorg Med Chem* 2002, **10**:3085-3112.

A good review summarizing the synthesis of tumour-associated glycopeptide antigens.

32. Takano Y, Habiro M, Someya M, Hojo H, Nakahara Y: **Preparation of core 2 type tetrasaccharide carrying decapeptide by benzyl protection-based solid-phase synthesis strategy.** *Tetrahedron Lett* 2002, **43**:8395-8399.

33. Brocke C, Kunz H: **Synthetic tumor-associated glycopeptide antigens from the tandem repeat sequence of the epithelial mucin MUC4.** *Synthesis* 2004:525-542.

34. Tanaka H, Adachi M, Takahashi T: **Efficient synthesis of core 2 class glycosyl amino acids by one-pot glycosylation approach.** *Tetrahedron Lett* 2004, **45**:1433-1436.

35. Hashihayata T, Ikegai K, Takeuchi K, Jona H, Mukaiyama T: **Convergent total syntheses of oligosaccharides by one-pot sequential stereoselective glycosylations.** *Bull Chem Soc Jpn* 2003, **76**:1829-1848.

36. Blixt O, Allin K, Pereira L, Datta A, Paulson JC: **Efficient chemoenzymatic synthesis of *O*-linked sialyl oligosaccharides.** *J Am Chem Soc* 2002, **124**:5739-5746.

37. Allen JR, Harris CR, Danishefsky SJ: **Pursuit of optimal carbohydrate-based anticancer vaccines: preparation of a multiantigenic unimolecular glycopeptide containing the Tn, MBr1, and Lewis(y) antigens.** *J Am Chem Soc* 2001, **123**:1890-1897.

38. Keding SJ, Endo A, Danishefsky SJ: **Synthesis of non-natural glycosylamino acids containing tumor-associated carbohydrate antigens.** *Tetrahedron* 2003, **59**:7023-7031.

39. Love KR, Seeberger PH: **Automated solid-phase synthesis of**
•• **protected tumor-associated antigen and blood group determinant oligosaccharides.** *Angew Chem Int Edit* 2004, **43**:602-605.

The synthesis of the Le^y-Le^x nonasaccharide, the Lewis X pentasaccharide and the Lewis Y hexasaccharide was efficiently performed on an automated synthesizer. Only five monomeric building blocks were necessary for the assembly of these target structures and the synthesis of the nonasaccharide was completed within 23 h.

40. Ferguson MAJ, Williams AF: **Cell-surface anchoring of proteins via glycosyl- phosphatidylinositol structures.** *Annu Rev Biochem* 1988, **57**:285-320.

41. Guo ZW, Bishop L: **Chemical synthesis of GPIs and**
• **GPI-anchored glycopeptides.** *Eur J Org Chem* 2004:3585-3596.

An excellent review summarizing recent approaches for the chemical synthesis of GPI anchors.

42. Lahmann M, Garegg PJ, Konradsson P, Oscarson S: **Synthesis of a polyphosphorylated GPI-anchor core structure.** *Can J Chem* 2002, **80**:1105-1111.

43. Pekari K, Schmidt RR: **A variable concept for the preparation of branched glycosyl phosphatidyl inositol anchors.** *J Org Chem* 2003, **68**:1295-1308.

44. Reichardt NC, Martin-Lomas M: **A practical solid-phase synthesis of glycosylphosphatidylinositol precursors.** *Angew Chem Int Edit* 2003, **42**:4674-4677.

45. Xue J, Guo ZW: **Convergent synthesis of a GPI containing an acylated inositol.** *J Am Chem Soc* 2003, **125**:16334-16339.

46. Xue J, Shao N, Guo ZW: **First total synthesis of a GPI-anchored peptide.** *J Org Chem* 2003, **68**:4020-4029.

47. Shao N, Xue B, Guo ZW: **Chemical synthesis of a skeleton**
•• **structure of sperm CD52 — a GPI-anchored glycopeptide.** *Angew Chem Int Edit* 2004, **43**:1569-1573.

The first report of the chemical synthesis of a complex and all natively linked glycopeptide–GPI conjugate. The preparation of a skeleton structure of the sperm CD52 antigen was achieved by first carrying out the separate synthesis of the protected glycopeptides and the GPI. After selective deprotection both segments were coupled by an amide bond to give the entire conjugate.

48. Schofield L, Hewitt MC, Evans K, Siomos MA, Seeberger PH: **Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria.** *Nature* 2002, **418**:785-789.

49. Seeberger PH, Soucy RL, Kwon Y-U, Snyder DA, Kanemitsu T: **A convergent, versatile route to two synthetic conjugate anti-toxin malaria vaccines.** *Chem Commun* 2004:1706-1707.

50. Hewitt MC, Snyder DA, Seeberger PH: **Rapid synthesis of a**
•• **glycosylphosphatidylinositol-based malaria vaccine using automated solid-phase oligosaccharide synthesis.** *J Am Chem Soc* 2002, **124**:13434-13436.

The automated synthesis of a hexasaccharide, which constitutes a promising malaria vaccine, has been carried out.

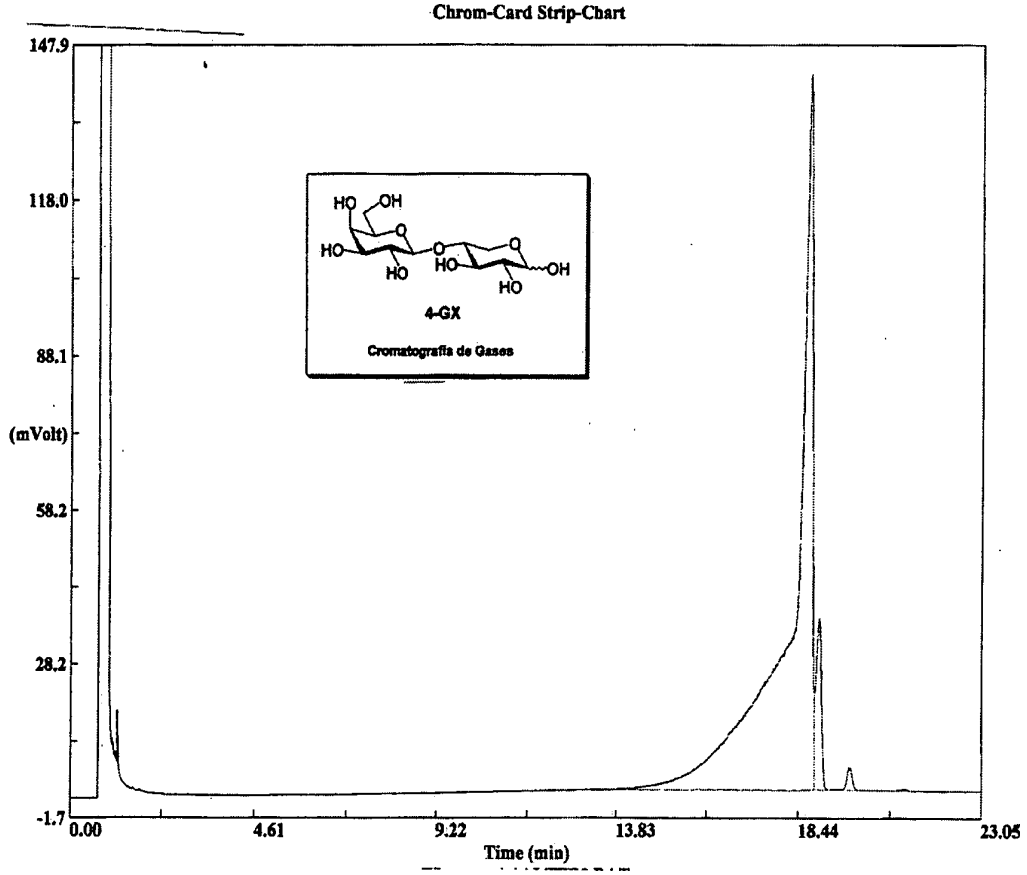
51. Lu J, Jayaprakash KN, Fraser-Reid B: **First synthesis of a malarial prototype: a fully lipidated and phosphorylated GPI membrane anchor.** *Tetrahedron Lett* 2004, **45**:879-882.

52. Lu J, Jayaprakash KN, Schlueter U, Fraser-Reid B: **Synthesis**
•• **of a malaria candidate glycosylphosphatidylinositol (GPI) structure: a strategy for fully inositol acylated and phosphorylated GPIs.** *J Am Chem Soc* 2004, **126**:7540-7547.

The synthesis and properties of a malarial GPI prototype and one variant are reported in this paper.

Exhibit D

Gas chromatogram of 4-galactosyl-xylose



Retention Time (min)	Peak Height (uV)	Area (.1*uV*sec)	Area % (%)	Plates (N)
18.700	138499	46240450	92.566	36622
18.928	33256	3266899	6.540	123711
19.713	4427	406455	0.814	104794
21.075	362	40018	0.080	81899
	192093	49953820		

Exhibit E

ear region of the adsorption isotherm, the retention time and the peak width, defined as the first absolute moment μ_1 and the second central moment μ_2, respectively, are functions of the quantities that characterize the chemical nature and texture of a solid packing [371-375]:

$$\mu_1 = f_1(K_s) \tag{175}$$

$$\mu_2 = f_2(K_s, k_a, \varepsilon', D_{int}^{-1}) \tag{176}$$

where

K_s = partition coefficient in GSC,
k_a = adsorption coefficient,
ε' = internal porosity of the particles with respect to the pore space (which, contrary to ε_0, the interparticle porosity, was not considered in eqns. (55) and (56),
D_{int} = effective diffusion coefficient of the separated substances in the pores of the solid.

Owing to the proportionality between K_s and the specific retention volume in GSC, V_g [eqn. (20)], the retention increases with increasing adsorption equilibrium constant, which in turn depends on the strength of the interaction of the compound to be adsorbed/desorbed with the solid phase. The differences in the K_s values for solutes with different chemical structures will be large, hence accomplishing the separation ($\mu_1 = f_1(K_s)$).

Strong adsorption invariably broadens the peak, as is apparent from eqn. (176) where K_s is involved, and as is confirmed by experience, i.e., it affects the efficiency and separability. A principal means of improving the peak width consists in the selection of adsorbents with a favourable pore size distribution, as μ_2 [eqn. (176)] depends on ε' and on the reciprocal of the diffusion coefficient D_{int} in the pores of the solid. In narrow pores, e.g., with pore diameters 10 nm, the Knudsen diffusion is predominant, i.e., collisions of sample molecules with the pore walls take place more often than with other sample molecules or with molecules of the carrier gas. This special diffusion, the rate of which increases linearly with increasing pore diameter, is very slow (e.g., for n-hexane at 10 Torr, $D_{kn} = 0.0202$ cm²/s, compared with the bulk diffusion coefficient for a hydrogen-n-hexane mixture of $D = 0.5148$ cm²/s [375, 376]). In wide pores, i.e., with pore diameters > 200 nm, the rate of diffusion is independent of the pore diameter and the effective diffusion coefficient is proportional to the bulk diffusion coefficient.

To summarize, there are two essential characteristics of adsorbents by which they can be classified: their chemical structure and their geometrical structure.

5.1.1. Classification According to Chemical Structure

Based on the chemical nature of the adsorbent surface, different kinds of interactions with different sample molecules can occur. *Kiselev*, whose proposed classification [377, 378-379] has been generally accepted and applied, subdivides adsorbents into three groups (I-III) and adsorbates into four groups (A-D).

Adsorbents of type I
Non-specific adsorbents, which do not have any functional groups or ions on the surface and hence are not capable of specifically interacting with adsorbates. The interaction with all types of sample molecules A-D proceeds non-specifically. Adsorbents of this type are saturated hydrocarbons (in crystalline or solid polymer modification or as a layer on a suitable supporting adsorbent), graphite or rare gas crystals. The most important adsorbent of this type is graphitized thermal carbon black (GTCB) which in its adsorption properties approaches an ideally non-specific adsorbent when prepared or pre-treated in a suitable manner.

The adsorbed molecules are arranged in such way that they contact the highest possible number of surface atoms.

Owing to their structure, which is similar to that of graphite, the inorganic adsorbents boron nitride (BN), and sulphides of some metals (e.g., MoS_2) can be included in this group [379, 380].

Adsorbents of type II

Specific adsorbents exhibiting positive partial charges localized on the surface. In addition to the dispersion interactions that occur on any adsorbent independent of its type, specific interactions develop, resulting in an orientation and localization of the adsorbate molecules at the sites with the highest charge. Especially salts, in which the positive charge is concentrated on cations of small radius whereas the negative charge is distributed over a relatively large volume, belong to this type (e.g., $BaSO_4$). Zeolites, the cations of which have small atomic volumes, whereas the negative charge is distributed over the inner bonds of a large complex anion formed from AlO_4- and SiO_4 tetrahedra, are also of this type [379].

However, the most significant representatives of this type are adsorbents with functional groups of protonated acids, such as hydroxylated silica gels, and with aprotic Lewis centres on the surface.

Sample molecules of type A (saturated hydrocarbons, rare gases) are adsorbed non-specifically, as only dispersion forces can become effective. Molecules of type B, C and D can be adsorbed specifically.

Type B include molecules with an electron density localized on some bonds or atoms: π-bonds (unsaturated and aromatic hydrocarbons); functional groups, the atoms of which exhibit unshared electron pairs (ethers, ketones, tertiary amines, pyridine, nitriles); high quadrupole moments (N_2 molecules)

The interaction between type B adsorbates and type II adsorbents occurs between the centres of higher electron density (sample molecule) and the positive charge of the adsorbent (for example, the acidic proton of hydroxylated silica gel or an appropriate cation (Li, Na, Mg, Ca) in zeolites or aprotic Lewis centre (Al, B) on the surface).

Type C molecules have a localized positive charge on a metal atom and the excess of the electron density is distributed over adjacent bonds (organometallic compounds). Because of the high reactivity of many organometallic compounds and of the risk of chemisorption, there have been only a few investigations of this interaction.

Type D molecules contain peripheral functional groups (OH, NH, etc.), the electron density of which is increased on one of the atoms (O, N) and diminished on the other (H). This group includes water, alcohols and primary and secondary amines. The specific interactions of type D adsorbates with type II adsorbents mainly involve forces between the positive charge centres of the adsorbent and the lone electron pairs of the O or N atoms of the sample molecules.

Adsorbents of type III

Specific adsorbents bearing centres of higher electron density on the surface. To this group belong polymers such as polyacrylonitrile, copolymers of vinylpyridine and divinylbenzene and polymers with C=O and —O— groups on the surface. Porous polymers based on styrene-ethylvinylbenzene, cross-linked with divinylbenzene, varied by applying different polymerization initiators with various functional groups, may also be included in this group, even if non-specific dispersion forces preponderate. Type III adsorbents include also crystal surfaces formed by anions, and especially chemically modified adsorbents or non-specific adsorbents covered by a dense monolayer of suitable substances, hence creating negative charge centres on the surface.

Adsorbents of type III interact non-specifically with adsorbates of type A and specifically

with types such as B, C and D by forces between the negative charge on the adsorbent's surface and the positive charge of the metal atom (C) or of the functional group's (OH, NH) proton (D) or of the dipole or an induced dipole (B).

5.1.2. Classification According to Geometrical Structure

We had stated that the geometry of the adsorbents influences especially the capacity term in eqn. (100). Hence the surface area should be as high as possible in order to increase this term. However, there are serious reservations. Increasing the surface area means either increasing the dispersity (with the consequence of an increase in heterogeneity due to the increasing contact points between the particles) or narrowing the pore diameters (with the disadvantage of Knudsen diffusion). The outcome of numerous investigations in this field, among which especially the work of *Kiselev* should be given prominence, has been that difficulties of this kind, having retarded the development of GSC for a long time, have been surmounted [379].

The role of the surface area can be derived from basic equations in Chapter 2. For infinitely small (zero) samples, the net retention volume V_N, under equilibrium conditions, is equal to the Henry constant of the adsorption equilibrium [379]:

$$V_N = K_H = \lim_{n_{ads.},\, c \to 0} \left(\frac{n_{ads}}{c} \right) \tag{177}$$

where c is the concentration of the sample in the gas phase. If we consider the total surface area of the adsorbent in the column, $m_A S_A$, where m_A = weight of the adsorbent and S_A = specific surface area of the adsorbent, we obtain (from eqns. (11) and (20)), neglecting the temperature,

$$V_S = \frac{V_N}{m_A S_A} = K_S ,$$

which is the adsorption coefficient (or the Henry constant referred to unit surface area of the adsorbent).

From $V_N = K_H$ we obtain the correlation of both constants K_H and K_S with the geometrical parameter:

$$V_N = K_H = m_A S_A V_S = m_A S_A K_S . \tag{178}$$

Hence V_N, the net retention volume, can be influenced by both the column parameters (weight of the adsorbent m_A) and the geometrical characteristic of the adsorbent, its specific surface area S_A. K_S, however, can be influenced by the chemical nature and structure of both the interacting adsorbent and adsorbate, expressed by analogy with eqn. (189a) as the partial molar adsorption enthalpy [381]:

$$\frac{\mathrm{d} \ln K_S}{\mathrm{d}T} = \frac{\Delta H_A}{RT^2} . \tag{179}$$

As ΔH_A, the partial molar adsorption enhalpy, changes only slightly with the temperature, we can write [379]

$$\ln K_S \approx - \frac{\Delta H_A}{RT} + \frac{\Delta S_A}{R} + 1 \tag{180}$$

or

$$K_S \approx \exp\left[\frac{\Delta S_A}{R} + 1 \right] exp\left[\frac{-\Delta H_A}{RT} \right], \tag{180a}$$

where ΔS_A = partial molar adsorption entropy of the adsorbate for the transition from the standard state of the gas volume with concentration c^0 into the standard adsorbate state with an adsorption concentration Γ^0.

Eqn. (180a) shows the exponential dependence of K_S on temperature, the third essential parameter in GSC, in addition to the adsorbent's chemical structure and geometrical structure. Eqns. (178) and (180a) demonstrate that even adsorbents with small specific surfaces areas permit the separation of weakly adsorbable gases, provided that the column temperature is decreased accordingly, hence increasing K_S and thus also its product with the surface area, $m_A S_A K_S$ [379]. The alternative, or better for completion when separating low boiling gases, is the use of adsorbents with small particle diameters and/or fine pores, hence increasing S_A and $m_A S_A$.

Kiselev and *Yashin* [377] classified adsorbents geometrically as follows.

Type 1 Non-porous adsorbents
Crystalline products with a smooth surface (sodium chloride, graphitized thermal carbon black, BN, MoS_2)
S_A values 0.1–12 m^2/g.

Type 2 Uniformly porous adsorbents with wide pores
Silica gels with pore diameters between 10 and 200 nm (but each silica gel product with narrowly distributed pore diameters!)
(Porasil, Spherasil) and phases bonded chemically on silica gel
(Durapak, etc.) as also some wide-pore styrene-divinylbenzene polymers (pore diameters 20–400 nm).

Type 3 Uniformly porous adsorbents with narrow pores
Molecular sieves (zeolites), carbon molecular sieves, porous glasses, porous polymers. Pore diameters 10 nm.

Type 4 Irregularly porous adsorbents
Active charcoal, alumina. Owing to the geometrical (and chemical!) heterogeneity [the pore diameters range from 2–20 nm (transition pores) up to >200 nm (macropores)], such adsorbents are not appropriate for GSC (with the exception of their use as enrichment materials), even though they were widely applied in the early years of gas chromatography.

This classification is based on the existence and size of the pores. Porous adsorbents differ from non-porous solids by a void structure shaped from a system of pores. This structure can be characterized, independent of the chemical composition of the adsorbent, by the following quantities [382]:

- Specific surface area S_A (geometric size of the pore wall area per gram of adsorbent).
- Specific pore volume V_p (total pore volume per gram of adsorbent).
- Mean pore diameter d_{50} (average diameter of 50% of the pores; this value is identical with the maximum frequency only for a homogeneous pore size distribution).
- Pore size distribution (distribution function $d(V_p)/d(d_{50})$).

They can be determined by gas chromatography [381], mercury porosimetry and reversed size exclusion chromatography [383]. Important for porous adsorbents is the ratio of their pore diameters to the diameters of the adsorbate molecules. If this ratio is high, i.e., the pore diameters are much larger than the molecule diameters, then the adsorption equilibrium is established rapidly. If the pore diameters are similar in size to the adsorbate molecules, then the adsorption rate depends on both the pore shape and the size of the molecules. In narrow pores the adsorbed molecule may interact with surface atoms of the opposite pore walls, and the exchange of molecules with the mobile phase is delayed. Hence the adsorption behaviour

Exhibit F

FRACTIONATION OF THE REACTION MIXTURE THROUGH A CARBON/CELITE COLUMN USING DIFFERENT SOLVENTS

In order to find the appropriate solvent system for the elution of the carbon/celite column, a typical reaction mixture was chromatographed using different water-alcohol mixtures as eluent. The results indicated that the volume of solvent was significantly reduced when a mixture of water:isopropanol was used as eluent.

Reaction conditions and results of fractionation:

a) Reaction (following the described procedure):

- Nitrophenyl galactopyranoside, 5g.
- Xylose, 25 g.
- β-galactosidase from *E. Coli.*
- Phosphate buffer (pH, 7.0), 330 mL.
- 37 °C.

b) Fractionation through carbon/celite column:

Once the reaction is finished, the crude mixture is fractionated through a carbon/celite column using mixtures of alcohols and water as the eluent. The results are summarized in the following Table.

Table

Eluent	Eluent gradient (ratio water:alcohol)	Total volume (litres) used
water:methanol	100:0 (initial) → 70:30(final)	12.9
water:ethanol	100:0 (initial) → 90:10 (final)	7.5
water:isopropanol	100:0 (initial) → 92:8(final)	5.8
water:isopropanol	98:2 (initial) → 95:5(final)	3.6